MELANOMA GENOMICS

Terrence P McGarty
Draft 2

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by

Terrence P McGarty

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Preface

This work is the result of looking at the various pathways of many cancers and also seeing that melanoma is currently responding to pathway control mechanism as was first accomplished in chronic myelogenous leukemia. Melanoma is a difficult but superficial cancer. It sits initially on the surface but rapidly spreads to a diverse set of organs, from lung, to brain, to bones. It is highly aggressive and to date has resisted almost all forms of treatment except early detection. In a sense with proper surveillance most melanomas should not evolve to a life threatening disease. The exception is nodular melanoma which starts with an aggressive vertical growth stage and grows to a metastatic capability rapidly even with significant surveillance.

The intent here was to frame the progression of melanoma in genomic systems terms, meaning understanding the intracellular genomic model as well as understanding the intercellular genomic model. The problem often with modeling cancers of various types is the dynamism in the discovery of new paths and factors. Genomic models are evolving and we have attempted to use the most extensive of effective techniques in establishing a model for this disease. We also suspect that the tools themselves will evolve and become more sophisticated and what we have tried to do herein is provide the toolset best available at the time.

Much of the work today falls into three camps: (i) those identifying the specific gene products which are present in melanoma, and thus trying to identify the corresponding gene aberration, (ii) approaches looking at pathways such as the Akt, PTEN path and the B-RAF, MEK path. Pathway identification may allow for multiple points of control in the event of failure in single point control, (iii) global intra-inter cellular signalling using the cancer stem cell paradigm, looking at the cancer as a distributed cellular phenomenon. The latter is clearly in its nascent stage. Our attempt herein is to build on the first two and develop some insight to the third.

This is not a clinical text nor is a text on fundamental bench methods or discovery This was written to look at the disease as a system and to develop systems models for the disease. Genomic systems models must incorporate the intra and inter cellular temporal and spatial dynamics. As such the sustainability of any such model will be predicated upon its ability to be both descriptive and predictive. We must be able to utilize the model to describe the disease in a consistent manner reflecting the facts as we experimentally know them and just as importantly to predict what will occur. In many ways this is applying an engineering methodology to understanding melanoma.

The major difficulty in writing a book of this type is that not all of the story is in. Our goal was to look at what others have done in a systems context. We use what the experimentalists have obtained and attempt to put structure and connectivity to it. The problem is that we may very well be missing some key elements which are yet to be uncovered. Thus we are establishing something which is in essence a moving target. Thus the results we obtain are methodological and conceptual and are not definitive. All that we look at is still a work in progress.

Such issues as the cancer stem cell, what makes the DNA express itself differently (mutations, micro RNA, methylation, all of the above, none of the above), cell surface receptors, inter-

cellular communications, all make for a moving target. But alas one must start somewhere. And that was the intent in putting these elements together.

We have taken a first cut at doing what we propose herein for prostate cancer. That was similar but in many ways fundamentally different. We can observe prostate cancer evolve in a slower manner from a melanoma. Melanoma, once it breaks loose, is in almost all cases deadly. I have seen hundreds of cases where because of delay in diagnosis the patient presents with lymphatic spread. That in almost all cases is a death sentence. Recent work on B-RAF has set a market akin to CML use of imatinib. These are targeted approaches to slow the process of proliferation. We seek to develop a framework to understand and possible provide useful insight.

This is a work of one who is fundamentally an engineer, a systems engineer. It is not a medical work nor a work by one expert at the bench. It is a work which relies on those expositions and tries to tie them all together using the tools of system engineering. One builds a model, tests it against facts, observes its deficiencies and goes back to the drawing board. That is the intent herein, to provide an alternative start at that process.

Also this is a draft document, and as such is a work in progress. It is subject to change, modification, editing, and may contain errors subject to correction. Comments are welcome.

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1 INTRODUCTION

Melanoma is an aggressive form of skin cancer, which generally is an uncontrolled growth of melanocytes and their progeny. Unlike so many other cancers which hide within the body, for the most part a melanoma generally sits on the surface of the epithelium, usually the skin, and shows itself to the observer. Melanomas are often very rapidly growing lesions. If on the skin, they can grow; (i) upward from the basal layer and be called a melanoma in situ, generally upon removal it is non-metastatic, (ii) horizontally which is called a superficial horizontal melanoma, but with metastatic potential, and (iii) a vertical growth stage below the basal layer which can be a very aggressive nodular form.

As indicated, melanoma is highly aggressive, and it both proliferates and metastasizes at an exceptionally high rate. The doubling time of melanoma cells is on the order of days, not months like an indolent form of prostate cancer. It is as aggressive as ovarian and pancreatic cancer, both of which have similar doubling times.

Melanoma is different from adenocarcinomas, the glandular like tumors we see in the prostate, breast, colon, pancreas, ovary, different than hematopoietic cancers of the bone and blood system, and of neurological tumors of the brain and nerve system. Melanoma starts with aberrant melanocytes, cells which originate from the neural crest, which normally inhabit the basal layer of the epidermis, and produce melanosomes, dark pigments, used for protection from the sun. However the melanocytes may suffer genetic changes which lead to growth and proliferation. Once this process starts it progresses at a rapid and never ending rate.

Melanoma like a few other cancers now has drug controllable means focusing on specific pathway alterations, the V600 BRAF mutation, and the control thereof. The medications blocking the aberrant gene are limited at this time but like the control of chronic myelogenous leukemia, CML, with kinase inhibitors, we have seen the first small steps with melanoma. What this does tell us is the understanding intracellular pathways are essential. Furthermore, as we shall demonstrate, controlling inter-cellular pathways is also critical.

1.1 OBJECT

The intent of this book is to look at melanoma in a systems framework. That is, we seek to look at the temporal and spatial dynamics of the cells and their ability to go from benign to malignant states and to focus on the specific elements which control this process. That is, we seek to determine the key state elements of a single cell and look at its dynamics and then to examine that cell in a total body over space and time. In simple terms, we see melanoma, and many cancers in general, as acting as a separate organism living off of the human host, while at the same time consuming parts of the host and replacing the host cells with malignant substitutes. This is a holistic view of cancer in that we try to consider all of the cells as part of a whole entity, which is genetically different that the host.

In this study we seek to answer the following questions:

1. What is the normal or homeostatic melanocyte look like? What controls the homeostatic cell, allowing new ones to be produced while eliminating older less functional cells. Melanocytes are different than keratinocytes in the skin. The keratinocyte starts in the basal layer and moves upwards and then dies off and the skin is shed. The melanocyte stays put. It does produce melanosomes, those dark pigments we can see reflected in tanned skin. But they have generally long life cycles.

2. What does a malignant melanocyte look like? What are the variables we should be concentrating on and how do we model its dynamic behavior? What controls the process and what is controlled by the process?

3. What are the "causes" of a malignancy? How can we measure the cause and what could be done to revert the process or at least stop the progression? Is it a genetic distortion, a methylation, a micro RNA issue or a set or combination of yet to be discovered processes?

4. What are the spatial characteristics of the malignant process? We look at this less from the perspective of the well-known metastatic progression but from the perspective of the intercellular spatial signalling. We know that melanoma cells are highly metastatic; they tend to spread aggressively to multiple organs. What causes that spread?

5. What are potential control points? What are steps in the process which can be activated or suppressed so that the malignancy can be brought under control? If we have a model for the temporal and spatial characteristics of an aberrant melanocyte derivative, then can we use knowledge of the control points to decrease the extent of the malignancy?

One of the problems we often face with cancers and melanoma is typical of this is the fact that not all melanomas are the same at the genetic level. There is no single constant genetic flaw that can be attacked and controlled. Oftentimes, there are different ones for different patients. We assume that we can examine melanoma cells on a gene or gene product basis and observe what is aberrant and from that tell what has to be controlled. However the aberrant element may itself be the result and not the cause.

From the systems approach we often look to see if the system has two important characteristics; observability and controllability. For our purposes we define observability as the ability, from measurement and observations available to us, to then determine the state of the system, namely its dynamic model, what makes it work. For our purposes, controllability is the capability to control the state of a system to some defined and desired level by means of the external control elements available to us. Thus we seek to ascertain if the models we are developing are observable and controllable. For then we can both justify the model on past data and then to use the model to make future predictions with some validity and control them to maintain homeostasis. That is the objective of system modeling in a genetic context.

Melanoma is in many ways a prototypical cancer yet it can be used in limited ways as a model for many other cancers despite its aggressive behavior. The specific issues relating to melanoma are:

1. Stem Cell Characteristics: As we shall discuss, there are stem cell models for cancer wherein a single stem cell can control all of is related malignant cells. The stem cell is the controlling and directing cell. Arguably f one removes the stem cell one eliminates the cancer, the remaining morphologically malignant cells require a stem cell and its absence sends the other cells into an apoptotic state. This is not the clonal theory, wherein all cancers come from a single aberrant cell. The stem cell paradigm assumes a clonal or eve a non-clonal beginning, it simply states that the cancer can progress only if a potent controlling and directing cell is present.

2. Intracellular Pathways: All cells have pathways which control the actions of the DNA and replications of the cells. The DNA in simple terms reduces various proteins which in turn may control the production of other proteins and which in turn control cell growth and replication. Loss of normal cell growth by loss of certain specific pathway elements is often the underlying "cause" of cancer. Intracellular pathways are small factories of the chemicals for cell homeostasis. Any change in normal DNA functioning may then result in aberrant cell behavior. It is essential to understand the pathways, to understand their dynamics and to understand what happens when they go unstable. It is also critical to understand how aberrant behavior can be assessed and managed. In a sense one way to deal with cancer is to manage it as a chronic disease, namely managing the aberrant cells.

3. Cause of Loss of Homeostatic Pathway Dynamics: Once we understand the pathway we can then assess what is normal and what is not normal. However it is critical to understand what happened to go from normal to abnormal and when that happened. In the case of melanoma there is significant data on the impact of ultraviolet light on the melanocyte. Yet melanoma may also occur in cells never exposed to light as well. Thus UV light may be one of multiple causes. There most likely is not a single pathway aberration leading to a melanoma but many, and there may not be a single cause but many as well. That will be the challenge as we proceed to understand the genomics and system dynamics of melanocytes as they progress to melanoma. Specifically there may very well be many types of melanomas, not a single type. The causes may be aberrant behavior during normal DNA reading and the creation of RNA. There may be a single nucleotide polymorphism, SNP, which all by itself makes a difference in RNA and thus a controlling protein. There may be one or several micro RNAs which force aberrant DNA readings, UV light may have caused a specific SNP, and there may be several SNPs which may be necessary before a distorted reading of the DNA results.

The whole issue of the cause of DNA being misread is a critical issue. For example loss of PTEN function may be the result of many different steps. Thus the melanomas from PTEN loss may be different even amongst themselves. This is the problem of using microarray techniques to determine prognostic markers for such cancers. The markers may be more an artifact of the causes than markers for the results.

4. Intercellular Pathways: The intercellular pathways are also of critical importance. In melanoma the surface proteins controlling cellular location at the basal layer, E cadherin, is a critical factor. Loss of that control is loss of location and thus purpose, the cell begins wandering. Add to this the issue of cellular communications with a stem cell and we now have a distributed cellular problem. We can take this one step further as demonstrated by recent research. The malignant melanocyte may actually use the benign cells in the environment to be activated and

provide growth ligands to further activate distant cells. The ligands activating the distant benign cells may be from a distant malignant melanocytic stem cell. Thus the melanoma cell may rely not only directly upon a distant cell to send growth signal, but can possibly be enlisting local benign cells to do the same. This means that there is a control at a distance via intercellular signalling proteins.

We have compared this in the past to the problem that Alan Turing addressed in examining zebra stripes. Let me give some details. "Why do zebras have stripes?" asked Alan Turing shortly before he died from a self-inflicted cause. The answer he provided in one of his last papers was, just at the time of Watson and Crick, because cells communicate with each other and turn genes on and off in an almost wave-like fashion. Turing hypothesized that there was some mechanism whereby cells spatially communicate, and that the communication results in the downstream cells to be activated in some progressive manner thus going from white to black in the case of a zebra. He hypothesized that the response was often nonlinear allowing for an either-or response.

We recently had written a paper regarding a similar phenomenon in the case of flower patterns and used the term Turing Tessellation to describe that phenomenon. That is we could show how patterns in flowers were the direct result of this flow of intercellular signalling and a non-linear receptor/activator. This means that cells produce proteins in an epigenetic manner and the proteins directly or as a result of the control they have on other molecules, communicate cell to cell, and this is what causes flower patterns.

In a recent paper in Cell[1] and reported Lester[2] the authors, Roesch et al, show that in Melanoma the stem cell theory, namely that it is just one single cell that goes wild is proven wrong in melanoma. What happens simply is that the cells "communicate" a la Turing, and then when proteins which control malignant cells start flowing they set off a chain reaction a la Turing and as we had shown in a paper a few years ago[3]. The cells pop up elsewhere. The metastasis is not from the single stem cell escaping but from the proteins going wild.

Lester writes:

Scientists at The Wistar Institute offer a new explanation for the persistent ability of melanoma cells to self-renew, one of the reasons why melanoma remains the deadliest form of skin cancer. The concept of the "dynamic stemness" of melanoma can explain why melanoma cells behave like both conventional tumor cells and cancer stem cells.

The researchers write in the May 14 issue of the journal Cell that - contrary to other published reports - melanoma does not appear to follow the hierarchic cancer stem cell model, where a single malignant "mother cell" both reproduces to produce new mother cells and differentiates to produce the bulk tumor population. Instead, all melanoma cells equally harbor cancer stem cell

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1 See Roesch, A Temporarily Distinct Subpopulation of Slow-Cycling Melanoma Cells Is Required for Continuous Tumor Growth, Cell, Volume 141, Issue 4, 14 May 2010, Pages 583–594**, and** http://www.sciencedirect.com/science/article/pii/S009286741000437X

2 http://www.medicalnewstoday.com/releases/188783.php

3 http://www.telmarc.com/References/2008%20Flower%20and%20Patterning%20in%20the%20Genus%20Hemerocallis%2004.pdf

potential and are capable of inducing new tumors. Their findings reveal the unique biology of melanoma, and suggest that melanoma requires a new therapeutic approach.

Lester continues:

The present study arose when Roesch discovered a link between the potential of JARID1B to decrease proliferation of melanoma cells and control stemness. He decided to see whether JARID1B could be a marker of slow growing melanoma stem cells. Initially, the results were promising, he says. JARID1B-expressing cells were slow-growing (as stem cells often are), and rare, accounting for about 5 percent of the tumor population. "At this point we were really happy because we thought we had found a cancer stem cell marker," Roesch said.

But then, two unexpected results occurred. First, Roesch found that all melanoma cells were equally capable of initiating tumors in a mouse model, regardless of whether they expressed JARID1B or not. Second, he found that JARID1B expression did not conform to the traditional model of stem cell development - cells that expressed the gene could turn it off, and cells that didn't, could turn it on. In other words, the gene's expression was plastic, rather than stable. "Basically, our data suggest that every melanoma cell can serve as source for indefinite replenishment of the tumor," said Roesch.

Thus it is the cell to cell flow, the building and decaying of protein concentrations which set off the malignant melanocytes.

The Roesch et al article summarizes the following key points:

- *The H3K4 demethylase JARID1B marks a subpopulation of slow-cycling melanoma cells*
- *The JARID1B+ subpopulation is required for continuous tumor maintenance*
- *Cells can lose or gain JARID1B expression and do not follow a stem cell hierarchy*
- *Tumor initiation is not necessarily linked with tumor maintenance*

This observation will undoubtedly change the "paradigm" in a Kuhn sense in viewing cancer. We have argued this for a few years now but it will take time for researchers to understand its implications in the full. For this we must thank Alan Turing.

1.2 WHAT IS A GENOMICS SYSTEM MODEL FOR CANCER?

A Systems Model for cancer is a mathematical embodiment of the elements of cancer generation and progression within the human. The Systems model is capable of being verified by experimental means and methods, and furthermore the model must be predictive. Albeit in a stochastic manner. A Systems Model of cancer genomics is thus a set of analytic expressions and/or models which are based upon physically determined relationships between and amongst the genes in a cell or collection of cells, their products such as proteins, and the elements which influence them and influenced by them, such that these expressions reflect in a repeatable manner the behavior of the cells in a local and extended micro environment using measurable and repeatable methods.

The Systems Model is an embodiment of the cancer cell, its dynamics, and its interactions over time. The Systems Model must be reliably predictive to a reasonable degree. The predictive characteristic is the "*sine qua non"* of any model. Failure to be predictive, and thus verifiable, makes such a model useless. Models also develop in an iterative manner.

A Systems Model builds upon the "bench work" of those who are examining the specific elements which are considered part of the cancer process.

1.2.1 Major Issues Regarding Cancer

In building a model one must address what the variables are and how the variable relate in space and time as well as with each other. For the purpose of this model the variables will be the products of genes, namely the proteins, and specifically the concentration of the proteins in cells and external to cells, namely the concentrations affecting cells in some broad area. Thus this is a spatio-temporal model which entails concentrations of proteins generated by genes.

In a normal cell, homeostasis, the day to day action of a cell, is the production of proteins due to transcription and translation and the replication of a cell or the death of a cell. Cells thus in a homeostatic world are in one of three states:

1. Reproducing
2. Dying through apoptosis
3. Manufacturing proteins for itself and its environment.

There may be a fourth state which is a metabolic state which may not entail the DNA but it merely the energy transfer to and from the cell. For the current model we will not include that.

Now any cell will have a way of going from one of the above three states to another. For our initial simple model we shall assume that this is accomplished via

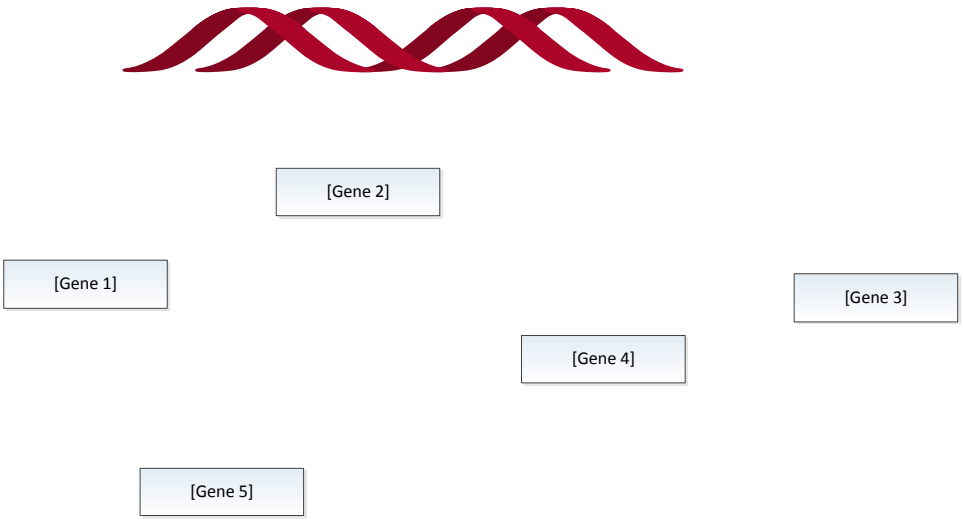
Now in simple terms cancer occurs as follows:

1. The normal homeostatic action of a gene is encumbered or destroyed.
2. Apoptosis does not occur, it may be blocked or its functionality reduced.
3. Cell reproduction, including impaired genetic production, occurs at a never ending rate.
4. The cells communicate outward allowing further degradation.

To understand this we first construct a simple model. This model will be useful in order to better understand where all the pathways we shall discuss fit in and more importantly what are we measuring and modeling in our system model.

First, within a cell we have DNA producing proteins whose concentrations are the controlling factors. As we shall describe later, the reaction rates are often controlled by concentrations, not just presence of a single protein strand. Thus the process of RAN to protein is a more complex step. One should also remember that there are activator and suppressor proteins which are

themselves the products of genes and again it is their concentration not just presence which results in the impact on the respective proteins produced.

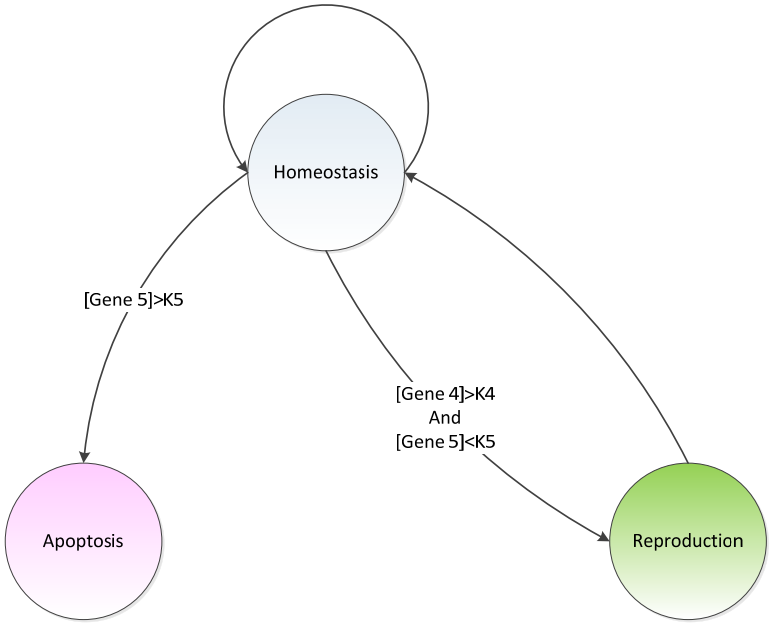


[Gene n] equals the concentration of the protein product of Gene n

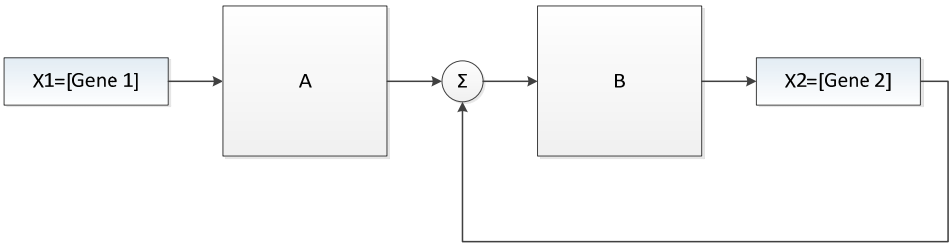
The DNA process of creating proteins is thus a complex dynamic system wherein the concentration of the proteins may be varying from time to time. Thus set of small but potentially significant variations are controlled within certain limits which are called the homeostatic limits.

Now we also have exogenous factors which may activate protein generation and we must include their effects as well, within the cell. We will discuss this later. For the most part the cell is just a little factory producing proteins and keeping them within certain bounds and using energy which is available to it for the purpose of allowing the factory to run.

However there are limits which if exceeded can turn the homeostatic somewhat steady state into one of two other possible states; apoptosis or cell death or cell proliferation that is starting the cell reproduction cycle. Below we show a simplistic cell which is one gene product concentration exceed a certain level we enter into cell death and if not but another exceeds a level we enter into cell proliferation. This simplified state diagram depicts what we often see but in considerably more complex systems.



Now the question is; how do we model the concentration dynamics so that we can understand when the cell goes from one state to another. We depict a simple example below of gene product concentrations.



$$\frac{d[Gene2]}{dt} = A[Gene2] + B[Gene1]$$

In this simple model we show that the dynamics of the gene concentrations can be modeled by a simple set of expressions. In reality these may be significantly more complex and reflective of the rate reactions of a highly integrated set of interdependent genes. One question that we must be able to answer is how one determines the rate constants that are reflected in the above simple model. That is the classic identification problem which has been studied extensively in the systems literature. More importantly one can readily apply Bayesian analysis to this process as well especially given that we have a system model upon which we are basing our identification process.

The simple model above can be expressed in more complex terms as below:

$$\frac{dx(t)}{dt} = Ax(t) + Bu(t)$$

where;

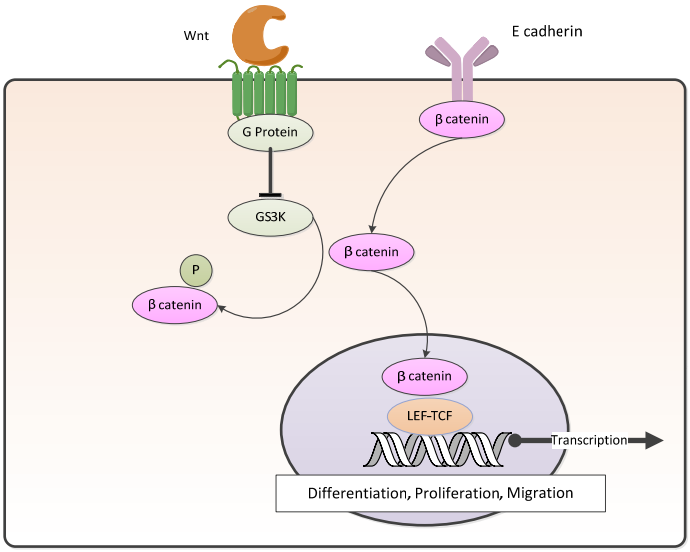
$$x = \begin{bmatrix} [Gene\ 1] \\ \dots \\ [Gene\ n] \end{bmatrix}$$

In addition this can be turned into a nonlinear model as well:

$$\frac{dx(t)}{dt} = f(x(t),t) + g(t)$$

Furthermore we can add random effects as well to reflect truly random factors and/or reflect possible uncertainties.

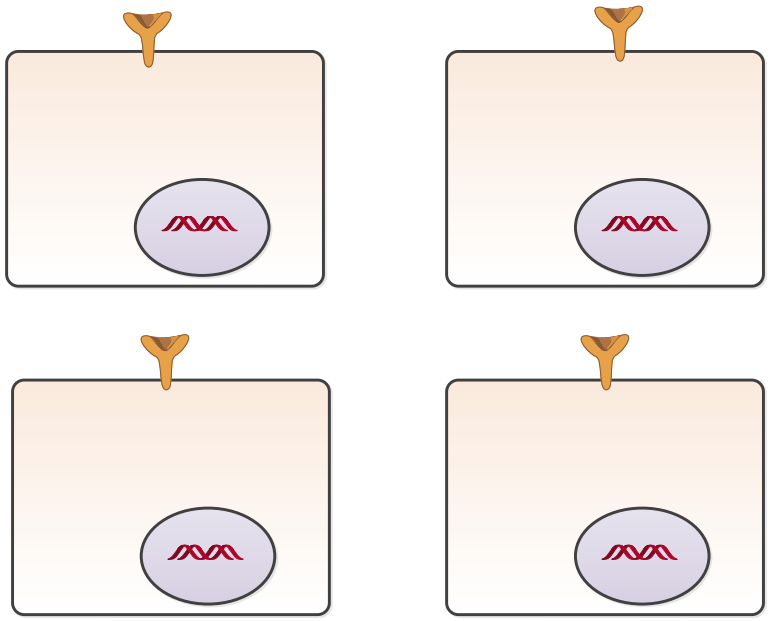
Now a typical cell set of pathways is shown below and this is reflective of a cell with a melanoma activate path. This is a clear example of some of the details we have been presenting above. The outside influence of the Wnt binding drives the internal pathways to have the cell proliferate. It is then the endless proliferation which is a major characteristic of the cancer cell. A significant other factor will be the loss of specificity or functionality.



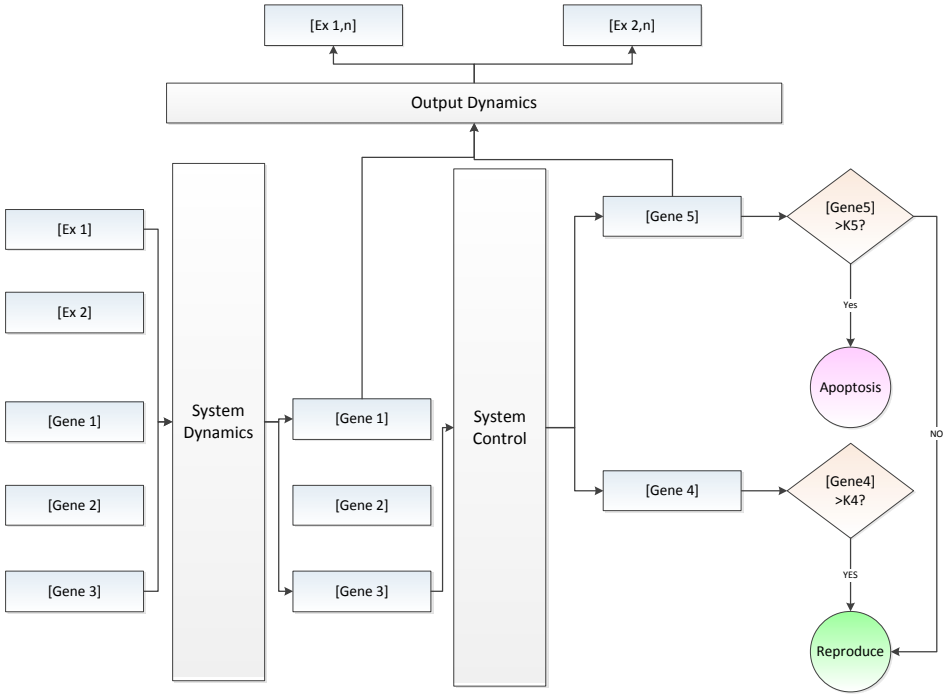
Adapted from Miller and Mihm NEJM July 2006

http://www.mmmp.org/MMMP/viewbiocard.mmmp?id=1301

Intercellular communications is facilitated by the communications via receptors. We demonstrated that above for Wnt and we depict that below for a collection of cells. This now details a spatio element of cells, namely the flow of messages and control from within a cell to across a large collection of cells.



In this simple 5 gene model we depict a more complete set of pathway dynamics in the figure below. Note we have 5 genes, but each gene may impact the expression of other genes. The impact may be positive or negative. The impact may cause cell death, apoptosis, or proliferations, mitosis. In this work we examine what genes produce what effects and we further examine what genes may have changes in them which cause an elimination of positive effects and an acceleration of negative effects.



Thus the above is representative of this five gene model where we have separate genes for apoptosis and proliferation. In this work we will attempt to examine all of the key gene pathways in a cell as well as those acting outside of a cell.

1.2.2 Understanding Cancer

Cancer is the loss of homeostasis in a particular type of cell or cell line. In one extreme sense it is the creation of a separate and independent collection of coordinated cells which become separate from the normal set of cells making up a human, and this alternate collection can through metastasis take over its host, resulting in death.

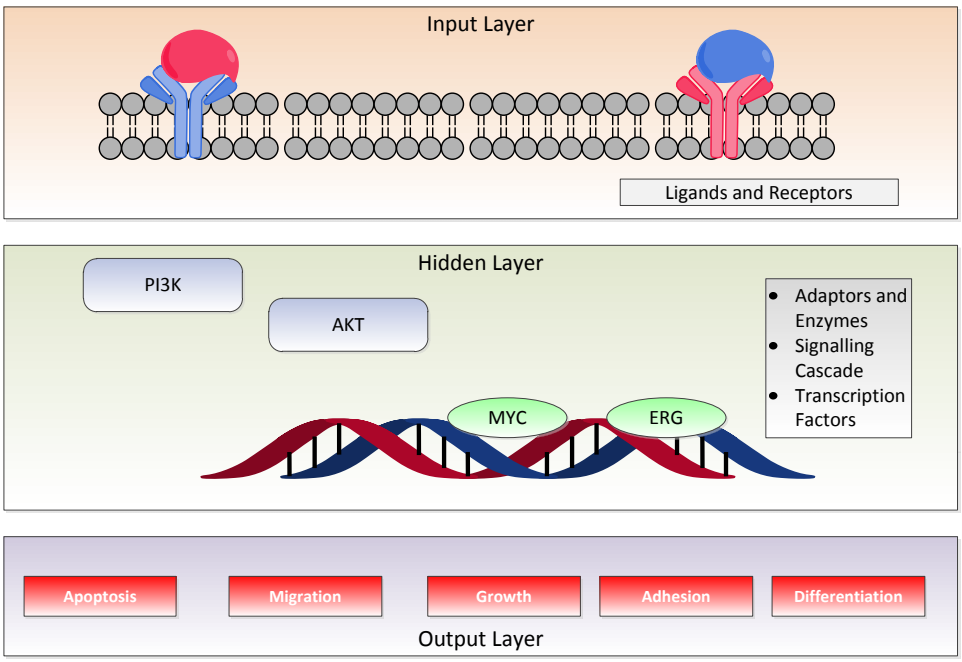
We demonstrate this below. We should not that in a cell, proteins, gene products, are often the major players. RNA may from time to time also be a significant factor which we shall discuss later. However, in a cell we have proteins which perform the following key functions as shown below:

1. Ligands: These are extracellular signalling proteins which we have already discussed. They flow between the cells, oftentimes crossing large distances.

2. Receptors: These are proteins that reside on the cell surface. They allow for binding of ligands on the outside and then activate pathways on the inside.

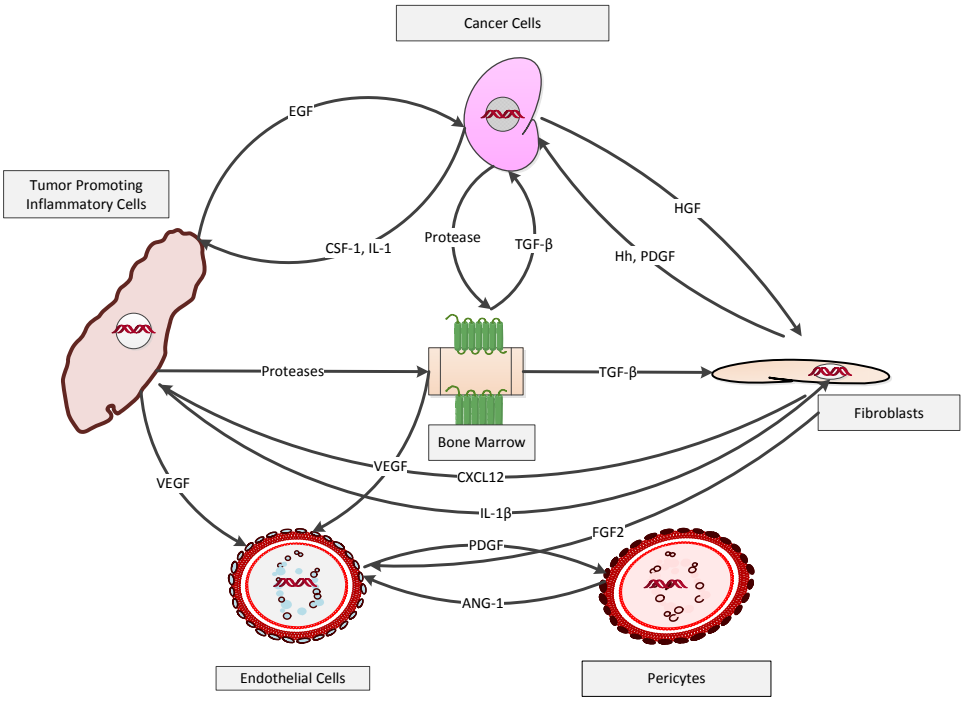
3. Pathway Elements: Inside the cell there are proteins which act in a cascade like manner carrying message from the surface to the nucleus. One protein after another in a highly complex positive and negative set of feedback loops are functioning.

4. Transcription Factors: Finally there are proteins which activate the transcription of genes into more proteins. These are the transcription factor genes. The loop is thus complete.

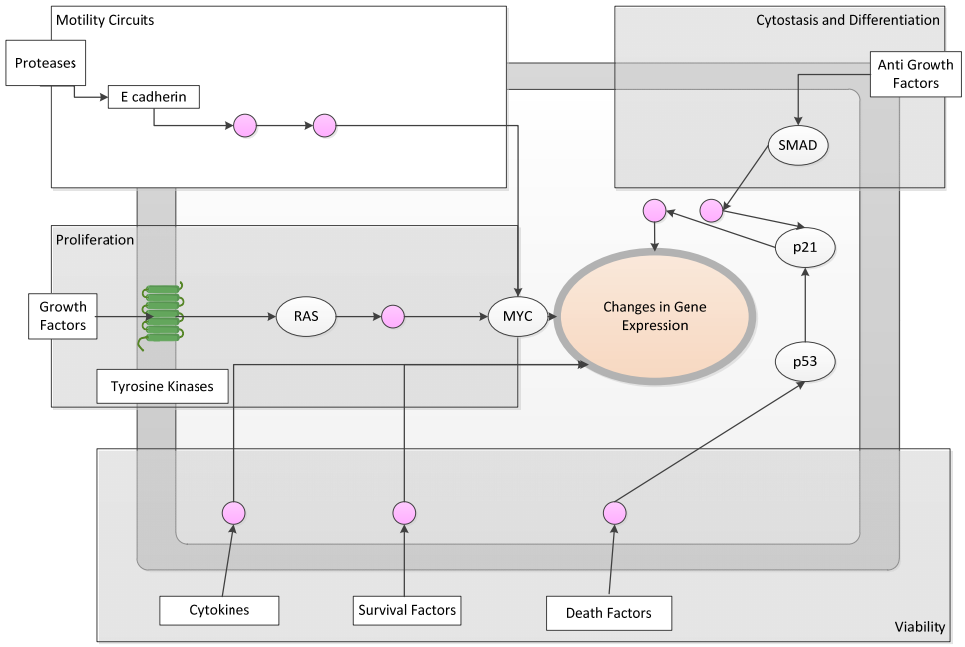


Thus in the above diagram we show these layers and the resulting functions. Cancer may be a result of loss of proper functionality of any one of hese elements.

In a series of papers by Hanahan and Weinberg, the authors have benchmarked progress in understanding cancer. We will go through some of their summary thoughts as a way to set up another view of the Systems Model approach.



The following depicts a functional flow of many of these circuits as described by Hanahan and Weinberg. This paradigm matches what we have been describing in the prior discussion, namely, exogenous factors impacting on internal pathways. The result is either a homeostasis or a cancerous cell. The Systems Model takes this one step further and details the actual interactions of the various gene products as measured in terms of the concentrations. The complexity associated with the spatio portions is deferred until later.

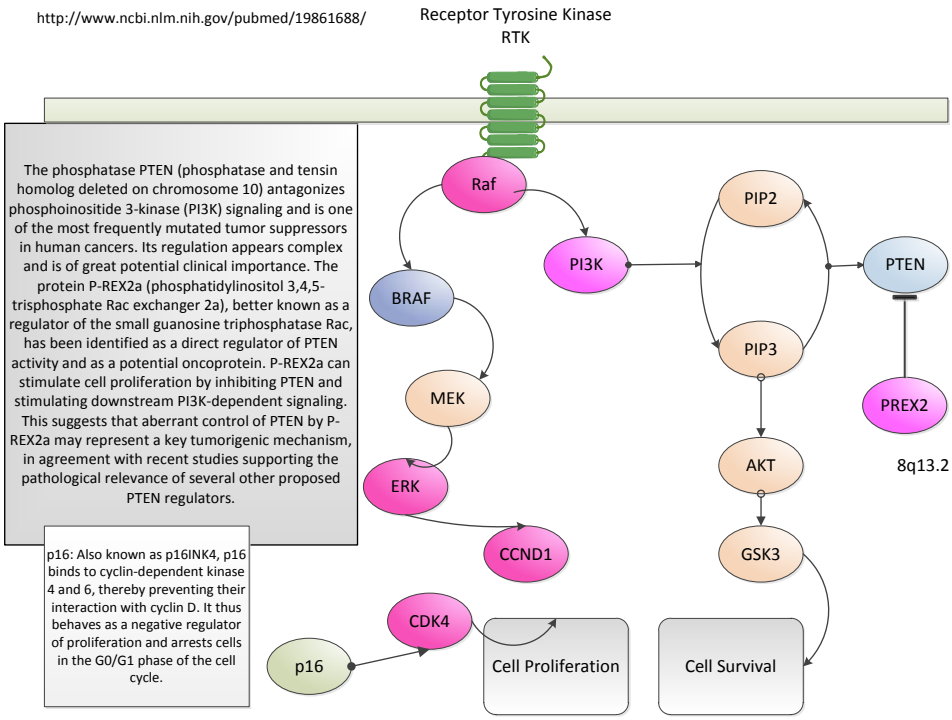


We now will show a specific melanoma pathway as an example. The following is the BRAF (V600 BRAF) pathway that we often see in melanoma. The process is somewhat simple:

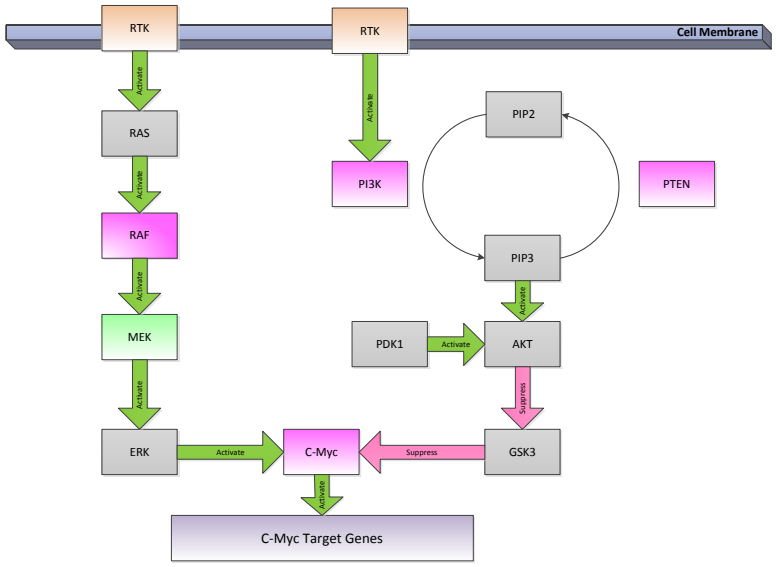
1. A growth factor attaches to a receptor, the RTK kinase shown in the figure.

2. This activates a pathway, a cascade of interacting proteins. RAF, MEK, ERK, CCND1, as well as PI3K and PTEN become active, some activating and some suppressing.

3. Transcription factors and CDKs, cycling dependent kinases, start off transcription and mitosis. Proliferation and growth progress.



We can now consider the following path. Here we have redrawn the above somewhat but have focused upon activating and controlling the transcription factor c-Myc. This specific path we shall see again many times.

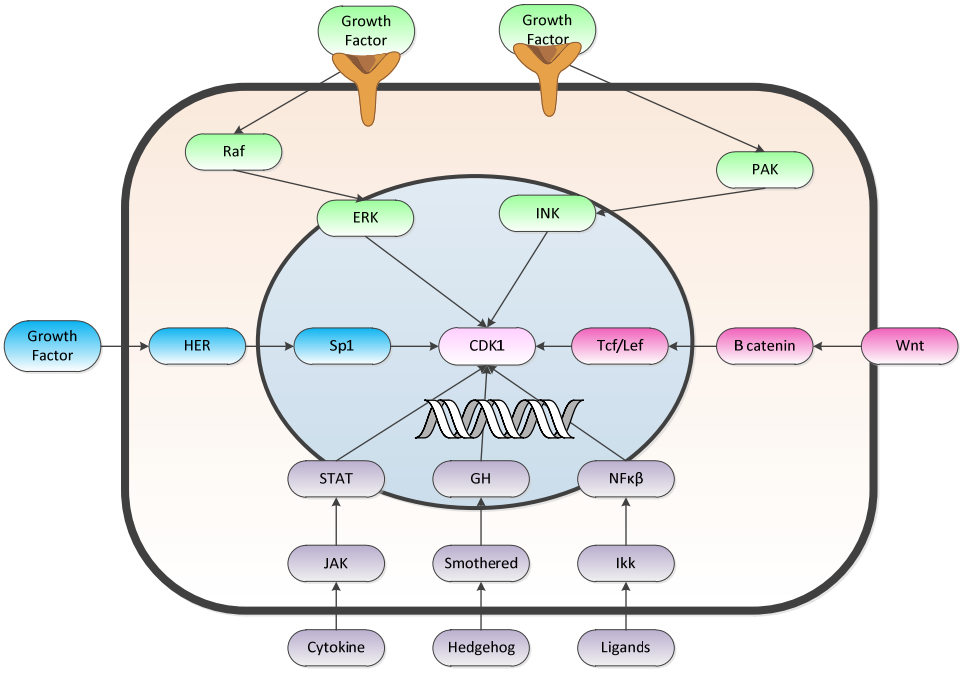


Now it is important to note that we have in the above pathway analysis assumed that somehow the proteins are always available. Also, that they are available in the right number, place and form. Many of those who develop models for these pathways and their dynamics do so assuming concentrations. We in act did so earlier herein. However in actuality the number of proteins of any one kind is clear denumerable, and thus actually counting them will be essential.

Concentrations make no sense at all. Thus models using reaction rate kinetics which inherently are averages and assume laws of large number type statistics fall apart on a cell by cell basis. We must recall this as we progress forward.

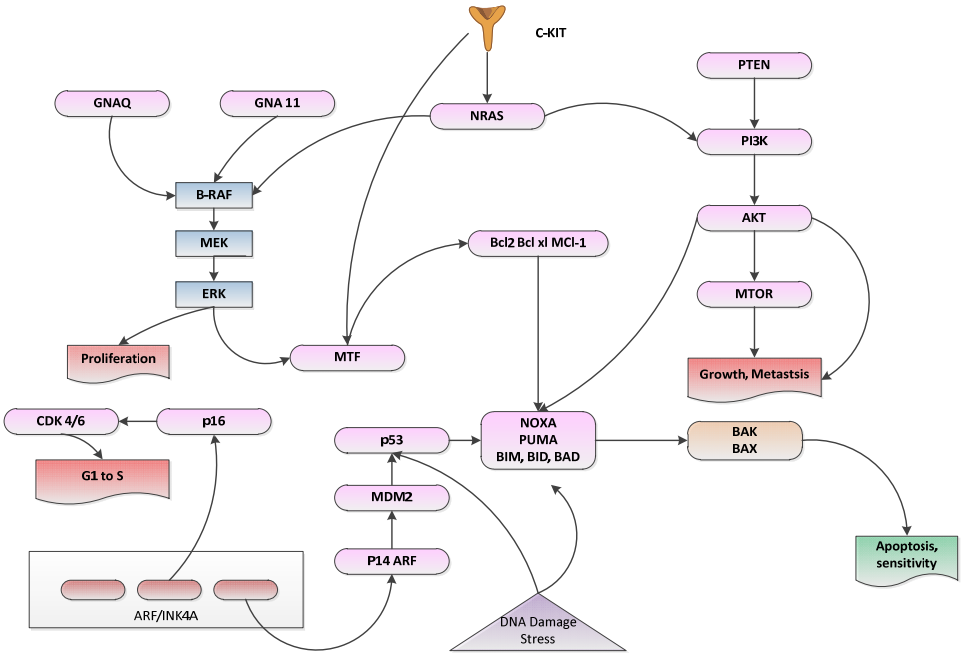
1.2.3 Pathways, Cross Talk and Stability

One of the challenging areas in the understanding of pathways is the fact that there is crosstalk between them. Although there are well define pathways they oftentimes can interact with one another creating a highly complex dynamic system.



The impact on the CDK transcription factor will be as we shall demonstrate is a key element in cell reproduction. CDKs are the fundamental driver of the mitotic cell division.

The above represents an amalgam of possible activated pathways. We now demonstrate the following:



This provides a brief overview of the pathway dynamics. We shall discuss each in considerable detail, especially when we examine the internal cell communications.

1.3 WHAT CAUSES CHANGE?

The major question we have when examining pathways is what causes the homeostatic pathway control to change. We know that a cell will reproduce when some exogenous factor is impacted on some cell receptor and then activates a pathway. The inherent homeostatic mechanism will return after the exogenous factor is removed. Thus a cell multiples until a point and then stops. But if a gene is blocked for a variety of reasons the stabilizing pathway is blocked and the cell may then continue to proliferate. This is often seen with Wnt pathway control problems.

The question then is what causes these gene expression changes. Note that we say "gene expression" change, for there are mechanisms such as miRNA which can extra the gene block expression at the mRNA level.

The following are possible causes of impaired transcription or translation:

1. Somatic Mutation
2. Germ line Mutation
3. Radiation induced mutation
4. Drug induced mutation
5. Methylation
6. miRNA interference

1.3.1 Weinberg Model

A generic model of cancer is shown below and is from Weinberg (2006). This is a highly simplified model but it shows the interactions between cell receptors which can be activated, cytoplasmic pathways, which control DNA transcription, and nuclear and DNA specifics which lead to cell proliferation and loss of apoptosis or death. Cancer cells have the ability to continue their proliferation and to avoid normal cell death. This is done via the instigation by external factors and the loss of cell death which would be a normal process.

Melanocytes have changes in their DNA which allows them to do a variety of new actions which in effect make them cancerous:

(i) They proliferate, namely they keep reproducing.

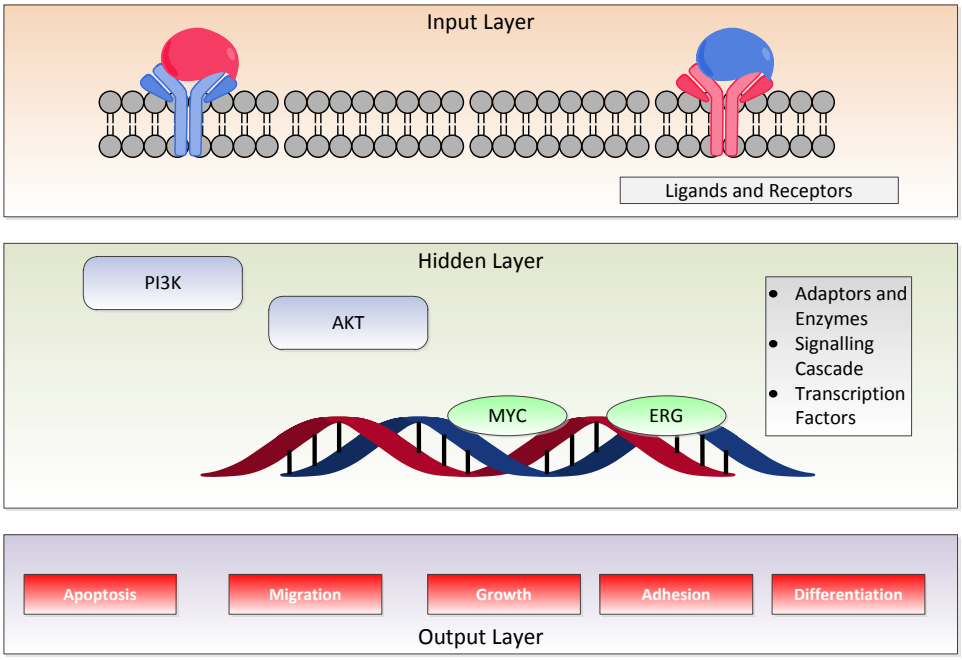
(ii) The loose the apoptotic behavior which is the normal limiting factor on cell life. They in effect become immortal.

(iii) They migrate. They move from where they are supposed to be to places where they are not supposed to be. This is a highly complex set of mechanisms whereby they can ignore the signals from the cells about them and establish a life of their own, disregarding the normal relationship between cells.

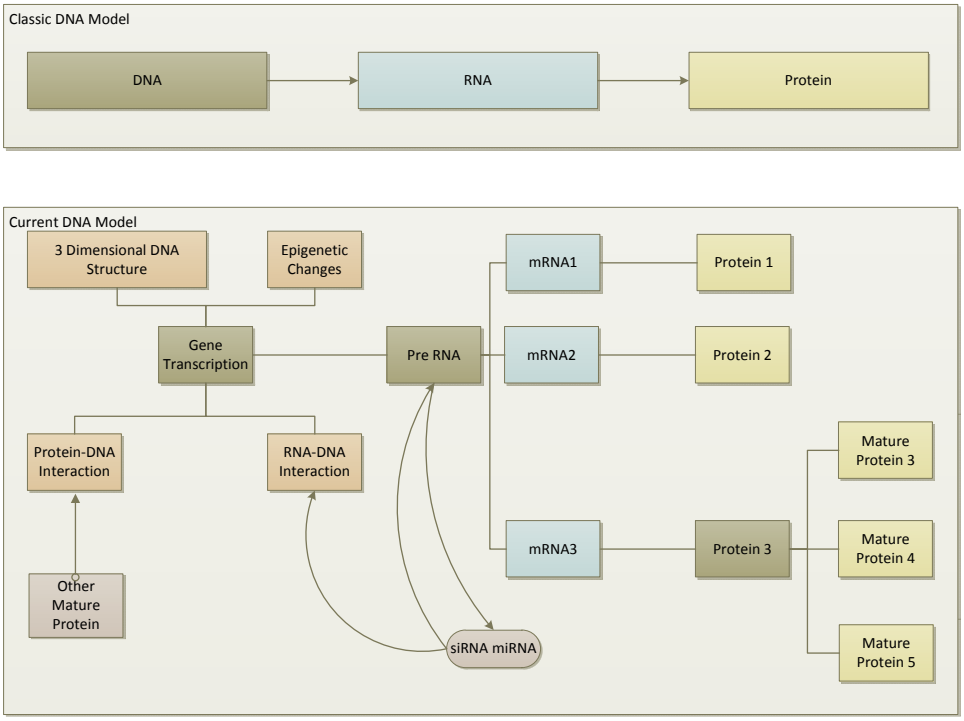
(iv) They have the ability to adhere to large clusters, namely the can create tumors or large masses of unregulated immortal cells.

(v) They lose the ability to differentiate. The cells take on abnormal shapes and often bizarre structures and yet can create a separate existence from that of the host.

The diagram is a repeat of what we had done before and shown below outlines the elements which we shall focus on.



This then leads to a changing understanding of DNA and its functions. The Figure below depicts the classic and the evolving model. The classic model is a simple one proposed by Watson and Crick in 1953. DNA goes to RNA goes to a protein. There is no interaction, no feedback, no noise, just a simple forward motion. By the 1960s Monod developed some more sophisticated ideas (see Weinberg) and this led eventually to a more complex view shown below the classic one. Here proteins interact with transcription factors and feedback is extensive. In addition we have other factors such as micro RNAs and epigenetic factors which alter the expression of the genes and thus the concentration of proteins.



In the above model, Weinberg's and the current view of pathways, we show names and contact points. In reality we have concentration of proteins, not just their very existence. The DNA is almost continuously producing various proteins, and the rate at which it does so depends on the concentration of other proteins and the reactions in the cell are themselves reactions where proteins are transformed by catalytic actions of other proteins. There is a complex enzymatic protein production through transcription and translation.

Thus in reality we may have, within any single cell, a dynamic as follows:

Let

$$p(t) = \begin{bmatrix} p_1(t) \\ . \\ . \\ . \\ p_n(t) \end{bmatrix} = \begin{bmatrix} Concentration\ p_1 \\ . \\ . \\ . \\ Concentration\ p_n \end{bmatrix}$$

then

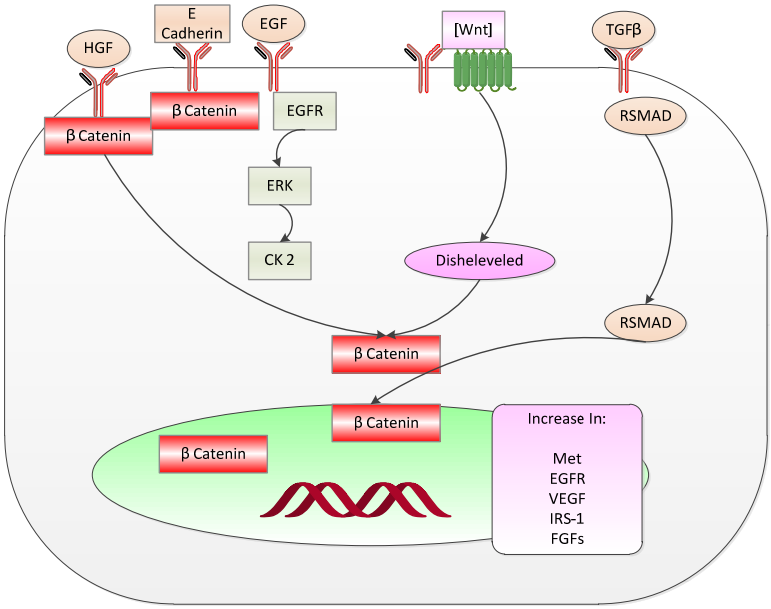
$$\frac{dp(t)}{dt} = f(p(t))$$

That is the concentrations of the proteins of interest are some complex intracellular dynamic process. In reality it may be very well more complex than this. It may include "noise" from methylation or micro RNAs, it may be dynamically changing from gene mutations, and it may be spatial in character, namely this may be true at point x but concentrations from adjoining cells at x+Δ may have a significant role to play as well say through cell surface receptors. This is the essence of systems modeling in genomics.

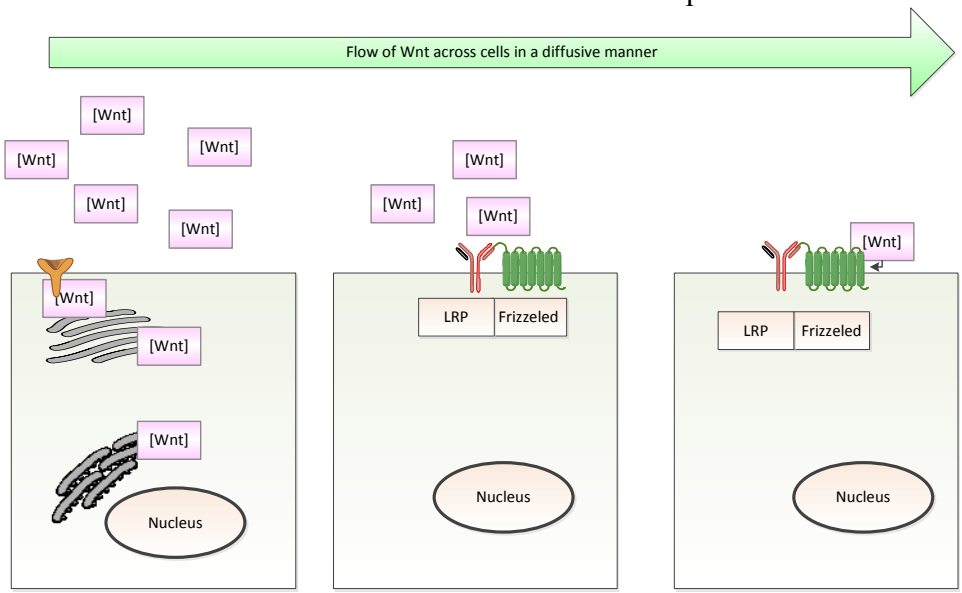
The above model focuses on intra-cellular pathways. We shall consider the inter-cellular as well but the main concerns in cancer are the proliferation and lack of natural death, apoptosis. The cell has an almost adaptive network of pathways which and change and increase its ability to survive and prosper. Normal cells understand their environment and thus can relate to what their proper role is as say a keratinocyte or melanocyte. They know their place and this knowledge is in many ways imbedded in the pathways and through the signals sent to it via the cell surface receptors.

1.3.2 Wnt: An Intercellular Communicator

One of the key communicating elements is the protein Wnt. We provide a brief introduction to it here because of both its singular importance as well as its basis for understanding intercellular communications and control.

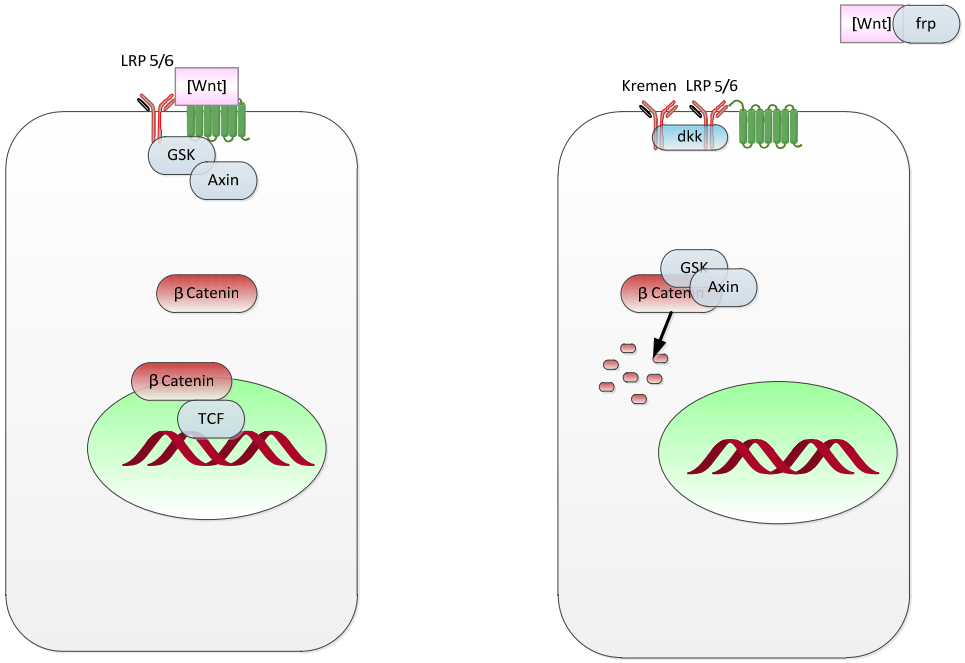


The increase in catenin is then a driver for an increase in transcription factors.



Wnt Generating Cell, is generated in Endoplastic Reticulum then up to Golgi Apparatus and then out via a receptor.

Now below we show the difference between an on and an off cell activated by Wnt. On the left we have the activated cell. Wnt is bound to the cell surface and in so doing activates a set of pathway cascades which in turn activate catenin which in turn activates transcription factors. Un activated the catenin gets ubuiquated via an ubiquitin breakdown and never is capable of transcription. The trouble can then begin in transcription.



Wnt Activated ON

Wnt Activated OFF

This provides a basis for understanding what are the key factors in the genomics of melanoma and cancer in general.

Wnt is but one of many such intercellular ligands used for communicating and activating. When we consider the cancer stem cell, we will also see that such intercellular communications from a control type cell can be a unique feature of many cancers. In addition it may also be a feature of normal cells as well.

1.4 OVERVIEW

We now will present an overview of the text and place each of the following chapters in the context of establishing a basis for the development of a systems model of melanoma. The following areas are covered:

Cancer Model and Principles: We review the general understanding of cancer models. We include such things as cancer metabolism, cancer pathway principles, and current model descriptions.

Skin Anatomy and Histology: In this chapter we discuss the issue of skin morphology at the microscopic level. This is only the slightest introduction to dermatopathology. It provides a minimal physical interpretation that will assist in understanding pathway effects.

Cell Cycle: Proliferation is one of the key factors in cancer, uncontrolled proliferation specifically. We examine mitosis in detail with specific emphasis on cell control proteins.

Intracellular Pathways: This is a detailed chapter on intracellular pathways from ligands to transcription proteins. We look at several dozen key proteins and also examine epigenetic factors such as miRNAs.

Extracellular Factors: Here we examine the extracellular pathways elements and the factors whose loss results in the diminution of cellular integrity.

Stem Cells: This is a critical concept, the stem cell. It goes to the heart of almost all cancers and it is still highly problematic. Simply the argument is that a few stem cells control everything, and that it is the stem cell which must be stopped. Unfortunately this is NOT what we examine when doing GWA, genome wide analysis, of a cancer. We examine a batch. We detail the principles here.

Prognosis, Management, and Exposure: In this Chapter we review the available gene markers and treatments using genetic based methods. We also discuss causes especially examining backscatter X-rays as an environmental example.

Epistemology: How to model something so that it does the right thing and asks the right question is at the heart of what we are trying to do. Such authors as Dougherty and also Winograd and Flores have examined the epistemological issue regarding highly technical areas; asking the right question. We spend a good deal of effort on these issues. For example, with the use of GWA, and microarrays, we have the ability to get billions of bits of information. We quite often have no idea what to do with it. Worse, researchers often announce the discovery of another gene. It may be purely coincidental. Causality and repeatability are lacking. That is what we try to present in this chapter.

Cancer Dynamic System Models: In this Chapter we develop the mathematical models we shall use in the final section.

Total Cellular Dynamics: In this Chapter we develop global cancer models. Specifically we develop a linkage between the intracellular dynamics, and the intercellular flow. This is a model showing the effects of “flow” in a Turing sense. We also present details of how one may measure the key parameters and then use the models in a control manner to control the distributed cancer process.

2 CURRENT ISSUES

We want to address certain current issue regarding cancer and melanoma in specific. We examine several specific areas of recent examination:

(i) A new view of cancer cell growth including both intra and inter cellular control mechanism,

(ii) Recent progress on specific protein targeting of mutated genes in cancer cells, building on the success which had been achieved in certain blood malignancies,

(iii) the cancer stem cell construct and its impact on melanoma.

We address these issues as a means to frame the discussion and to establish some insight to the model approach we will try to develop.

2.1 A NEW VIEW OF CANCER CELL GROWTH

We begin by referring to a recent paper by Hanahan and Weinberg (Hallmarks of Cancer: The Next Generation, Cell, 2011) which has some interesting observations on what has been developed in the area of cancer research. As they state at the outset:

The hallmarks of cancer comprise six biological capabilities acquired during the multistep development of human tumors. The hallmarks constitute an organizing principle for rationalizing the complexities of neoplastic disease. They include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis. Underlying these hallmarks are genome instability, which generates the genetic diversity that expedites their acquisition, and inflammation, which fosters multiple hallmark functions.

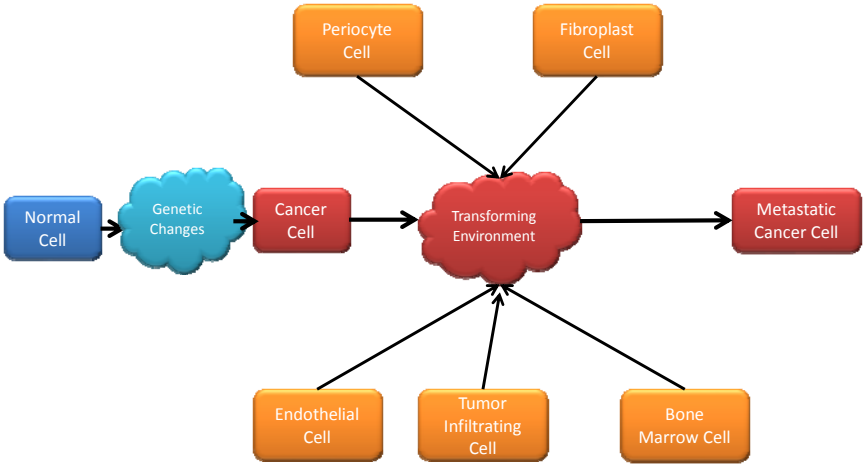
Conceptual progress in the last decade has added two emerging hallmarks of potential generality to this list—reprogramming of energy metabolism and evading immune destruction. In addition to cancer cells, tumors exhibit another dimension of complexity: they contain a repertoire of recruited, ostensibly normal cells that contribute to the acquisition of hallmark traits by creating the ‘‘tumor microenvironment.’’

Another dimension of complexity is not represented in this simple schematic: both neoplastic cells and the stromal cells around them change progressively during the multistep transformation of normal tissues into high-grade malignancies. This histopathological progression must reflect underlying changes in heterotypic signaling between tumor parenchyma and stroma. Such stepwise progression is likely to depend on back-and forth reciprocal interactions between the neoplastic cells and the supporting stromal cells, …. Thus, incipient neoplasias begin the interplay by recruiting and activating stromal cell types that assemble into an initial preneoplastic stroma, which in turn responds reciprocally by enhancing the neoplastic phenotypes of the nearby cancer cells.

The cancer cells, which may further evolve genetically, again feed signals back to the stroma, continuing the reprogramming of normal stromal cells to serve the budding neoplasm; ultimately signals originating in the tumor stroma enable cancer cells to invade normal adjacent tissues and disseminate. This model of reciprocal heterotypic signaling must be extended to encompass the final stage of multistep tumor progression—metastasis ...

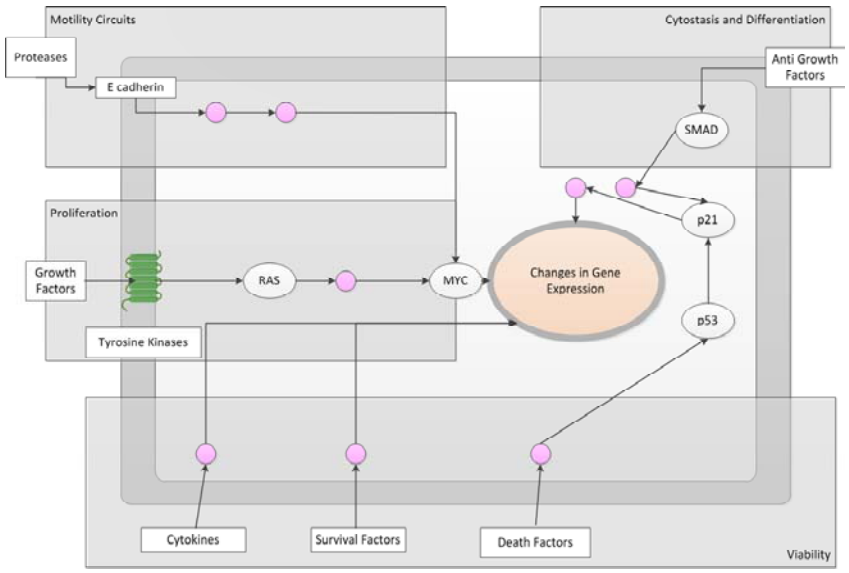
The circulating cancer cells that are released from primary tumors leave a microenvironment created by the supportive stroma of such tumors. However, upon landing in a distant organ, these cancer cells encounter a naive, fully normal, tissue microenvironment. Consequently, many of the heterotypic signals that shaped their phenotype while they resided within primary tumors may be absent in sites of dissemination, constituting a barrier to growth of the seeded cancer cells. Thus, the succession of reciprocal cancer cell to stromal cell interactions that defined multistep progression in the primary tumor now must be repeated anew in distant tissues as disseminated cancer cells proceed to colonize their newfound organ sites.

The model presented can be reduced to some simple descriptions, albeit a major change from a decade prior. The model is shown below:



Note that we have two different steps. The classic first step, the Vogelstein model, takes the normal cell via some genetic change into a cancer cell. The model here requires some genetic change where loss of the control mechanism for growth or death are altered. Understanding normal pathways we can see that if we lose a genetic element associated with some pathway we can lose the control elements which would result in a cancerous cell. However that does not in and of itself lead to a metastatic type of cancer, it may merely lead to a cell which grows more than it should.

When we examine the cancer cell we see a multiple set of pathways and a corresponding multiple set of means in which the pathway control of homeostasis can be degraded to create a cancer cell. We show such a model below which is a modification of what the author's present.



This model is an example of the classic pathway models which we have discussed earlier . Each of the pathways are triggered by some external stimulus and one often wondered what happened to do this?

To answer this question is the context of a larger model the authors report on a volume of research which has led to some key answers to this question. The authors consider six cell classes. They are:

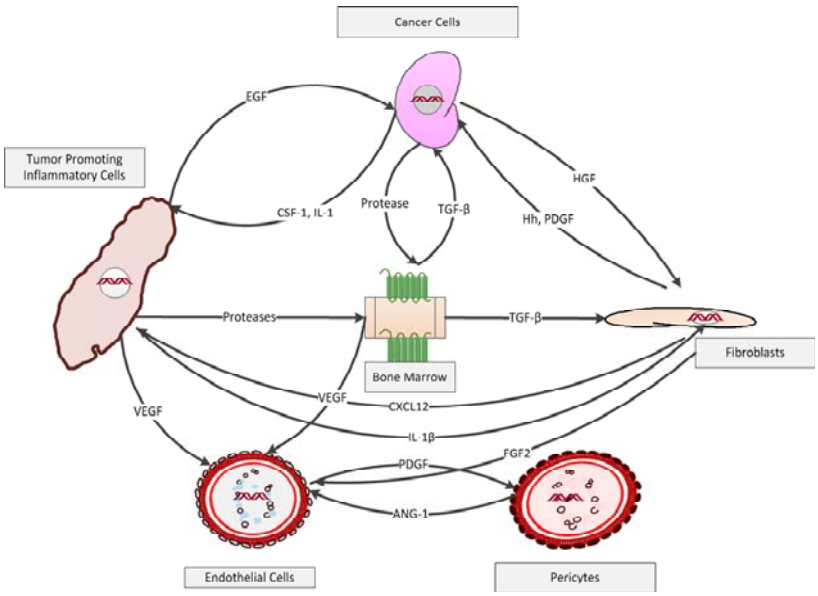
1. Cancer Cells – all the developing cancer cells
2. Bone Marrow – cells in the bone marrow
3. Tumor Promoting Inflammatory Cells
4. Endothelial Cells – thin layer of cells inside the blood vessels
5. Pericytes – connective tissue cell in blood vessels
6. Fibroblasts – generates the intercellular fabric

Now the cells communicate via the following signalling proteins, each with its receptor. They are:

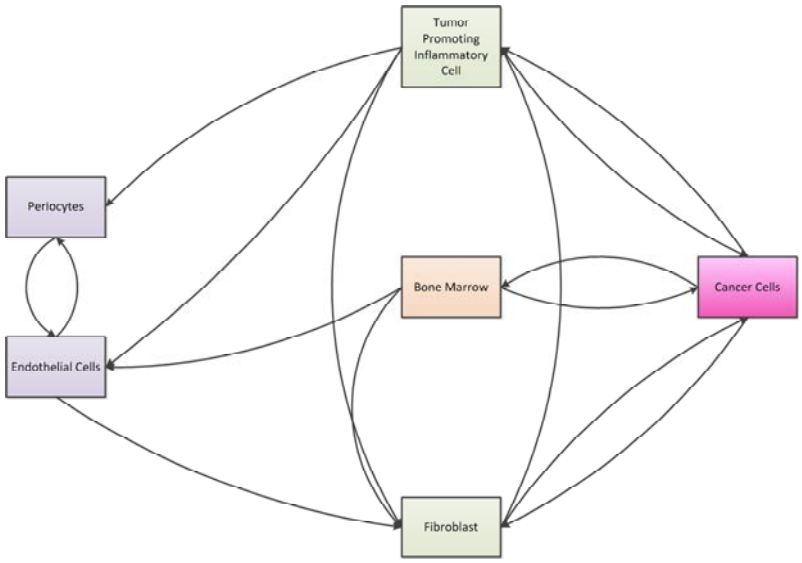
1. EGF- Epidermal Growth Factor attached to EGFR (receptor) and initiates cell growth.
2. HGF – Hepatocyte Growth Factor acts on endothelial cells and other cells
3. PDGF – Platelet Derived Growth Factor for blood vessel formation and is also a powerful mitogen.

4. VEGF – Vascular Endothelial Growth Factor produced by blood vessels and induces growth of new endothelial cells.
5. TGF-β – Tumor Growth Factor stimulates cell growth and tumor growth.
6. IL-1β – Interleukin 1 B important mediator of the inflammatory response, and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis.
7. Ang-1 – Angiopoetin 1 promotes angiogenesis
8. CXCL-12 – Chemokine Ligand 12, This gene product and its receptor CXCR4 can activate lymphocytes and have been implicated in the metastasis of some cancers such as breast cancer.
9. Protease - involved in angiogenesis
10. CSF-1 – Colony Stimulating Factor 1 a cytokine that controls the production, differentiation, and function of macrophages

We depict the result below:



Now we can reduce the above graphic to a system model which we show below. Here we show cells and driver stimuli from cell to another cell. We have a very complex network where the start of the process is the genetic change in a normal cell which becomes a cancer cell. This model as can readily be seen expands well beyond the cancer cell itself. As we have been discussing, in the cancer cell we have pathways which have been altered. Then outside the cell we have an environment which facilitates the cancer cell to proliferate. The question is does the cancer cell communicate to these other cells and use them as symbiots in the process of its proliferation and immortality? If so must one then have to include these cells very dynamics in a system model?



We can now look at the dynamics of this complex process. We can specify for example the concentrations of the products or cells as follows:

$$x_1 = [Cancer\ Cells]$$
$$x_2 = [Tumor\ Producing\ Inflammatory\ Cells]$$
$$x_3 = [Bone\ Marrow]$$
$$x_4 = [Fibroblast]$$
$$x_5 = [Endothelial\ Cells]$$
$$x_6 = [Pericytes]$$

Now one must remember that if we have no cancer cells then the process may continue without a malignancy. However if the cancer cell is developed then this model may represent the development of the cancer in the related micro environment.

Thus we can state:

$$\frac{dx(t)}{dt} = Ax(t)$$

where

$$x(t) = \begin{bmatrix} x_1(t) \\ \dots \\ x_n(t) \end{bmatrix}$$

The matric A relates the links which have been described. Inherent in this is also the reaction rate dynamics which we have linearized.

Now the process may go as follows:

1. In a benign state there is no cancer cell so that the system functions with the initial condition being zero for cancer cells.

2. Apart from the cycle, something happens inside a normal cell to alter its internal pathways, say from a genetic change, a methylation, some microRNA or the like.

3. This change then alters, activates, or deactivates a set of pathways which now can thrive in this environment.

4. The environment then becomes a positive feedback factor in the growth and dissemination of the cancer cells.

There are many interesting questions which may arise from this view. First the body itself propagates the cancer cells. Thus rather than just attacking the cancer cells we may consider attacking or modifying this supportive environment.

The authors conclude:

*Yet other areas are currently in rapid flux. In recent years**, elaborate molecular mechanisms controlling transcription through chromatin modifications have been uncovered, and there are clues that specific shifts in chromatin configuration occur during the acquisition of certain hallmark capabilities** …. Functionally significant epigenetic alterations seem likely to be factors not only in the cancer cells but also in the altered cells of the tumor-associated stroma. It is unclear at present whether an elucidation of these epigenetic mechanisms will materially change our overall understanding of the means by which hallmark capabilities are acquired or simply add additional detail to the regulatory circuitry that is already known to govern them.*

Similarly, the discovery of hundreds of distinct regulatory microRNAs has already led to profound changes in our understanding of the genetic control mechanisms that operate in health and disease. By now dozens of microRNAs have been implicated in various tumor phenotypes … and yet these only scratch the surface of the real complexity, as the functions of hundreds of microRNAs known to be present in our cells and altered in expression in different forms of cancer remain total mysteries. Here again, we are unclear as to whether future progress will cause fundamental shifts in our understanding of the pathogenetic mechanisms of cancer or only add detail to the elaborate regulatory circuits that have already been mapped out.

Finally, the circuit diagrams of heterotypic interactions between the multiple distinct cell types that assemble and collaborate to produce different forms and progressively malignant stages of cancer are currently rudimentary. In another decade, we anticipate that the signaling circuitry describing the intercommunication between these various cells within tumors will be charted in

far greater detail and clarity, eclipsing our current knowledge. And, as before … we continue to foresee cancer research as an increasingly logical science, in which myriad phenotypic complexities are manifestations of a small set of underlying organizing principles.

The three points in their conclusion are worth merit:

1. Epigenetics and the complexity of reactions

2. Micro RNAs and their overall influence

3. Heterogeneous pathway dynamics, as we have outlined, and their complexity.

The major effort to date was to understand intracellular pathways. Now we can combine them with extracellular, or spatial pathways. This reminds us again of the work by Turing, the old zebra stripe issue.

Micro RNA and epigenetics can become an issue akin to adding noise but what type. Their behavior is more akin to randomness at least as is currently understood.

Having these papers provides a wonderful baseline worth reviewing a decade at a time. Over the past forty years there has been a great deal of work regarding melanoma. Rosenberg at NCI had led the effort for many of the studies focusing on the use of the immune system.

2.2 CANCER METABOLISM: AN EXAMPLE OF COMPLEXITY

When discussing the Total Cellular Dynamics model we often encounter the question of where does the cancer cell get its growth energy from? Does it seek paths which offer it the richest environment for expansion or are there other mechanisms that make this unnecessary. We briefly examine the Warburg Hypothesis. Warburg conjectured that cancer is caused by damage to respiration, oxidation, and the cells getting energy via fermentation.

As Warburg noted in 1956:

Cancer cells originate from normal body cells in two phases. The first phase is the irreversible injuring of respiration. Just as there are many remote causes of plague-heat, insects, rats-but only one common cause, the plague bacillus, there are a great many remote causes of cancer-tar, rays, arsenic, pressure, urethane- but there is only one common cause into which all other causes of cancer merge, the irreversible injuring of respiration.

The irreversible injuring of respiration is followed, as the second phase of cancer formation, by a long struggle for existence by the injured cells to maintain their structure, in which a part of the cells perish from lack of energy, while another part succeed in replacing the irretrievably lost respiration energy by fermentation energy. Because of the morphological inferiority of fermentation energy, the highly differentiated body cells are converted by this into undifferentiated cells that grow wildly-the cancer cells

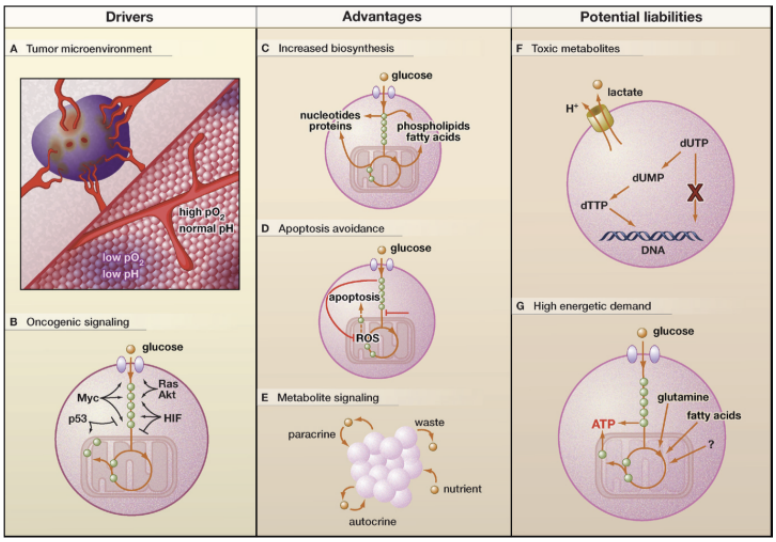
Although the Hypothesis was and is consistent with observations the cause and effect are highly suspect. Yet it does pose the issue of energy infusion in cancer cells.

As Hsu and Sabatini remark concerning Warburg:

It is hard to begin a discussion of cancer cell metabolism without first mentioning Otto Warburg. A pioneer in the study of respiration, Warburg made a striking discovery in the 1920s. He found that, even in the presence of ample oxygen, cancer cells prefer to metabolize glucose by glycolysis, a seeming paradox as glycolysis, when compared to oxidative phosphorylation, is a less efficient pathway for producing ATP (Warburg, 1956). The Warburg effect has since been demonstrated in different types of tumors and the concomitant increase in glucose uptake has been exploited clinically for the detection of tumors by fluoro-deoxy-glucose positron emission tomography (FDG-PET).

Although aerobic glycolysis has now been generally accepted as a metabolic hallmark of cancer, its causal relationship with cancer progression is still unclear. In this Essay, we discuss the possible drivers, advantages, and potential liabilities of the altered metabolism of cancer cells. Although our emphasis on the Warburg effect reflects the focus of the field, we would also like to encourage a broader approach to the study of cancer metabolism that takes into account the contributions of all interconnected small molecule pathways of the cell.

They demonstrate this in the Figure below showing pathway elements and advantages and disadvantages of such signalling:



And Hsu and Sabatini also demonstrate that this pathway control also controls apoptosis:

In addition to involvement in proliferation, altered metabolism may promote another cancer-essential function: the avoidance of apoptosis. Loss of the p53 target TIGAR sensitizes cancer cells to apoptosis, most likely by causing an increase in reactive oxygen species (Bensaad et al., 2006). On the other hand, overexpression of glyceraldehyde-3-phosphate dehydrogenase

(GAPDH) prevents caspase-independent cell death, presumably by stimulating glycolysis, increasing cellular ATP levels, and promoting autophagy (Colell et al., 2007). Whether or not GAPDH plays a physiological role in the regulation of cell death remains to be determined.

As Vander Heiden and Sabatini state:

In principle, the metabolic dependencies of cancer cells can be exploited for cancer treatment. For instance, a large fraction of human cancer is dependent on aberrant signaling through the PI3K/Akt pathway, and agents that target PI3K and various downstream signaling molecules are now in clinical trials.

The growing evidence that activation of PI3K causes increased dependency on glycolysis suggests that these agents may exert some of their effect by disrupting glucose metabolism. Drugs targeting key metabolic control points important for aerobic glycolysis, such as PK-M2 or LDH-A, might also warrant investigation as potential cancer therapies. In addition, the drugs developed to target metabolic diseases such as type 2 diabetes may have use in treating cancer. A number of retrospective clinical studies have found that the widely used diabetes drug metformin may offer a possible benefit in cancer prevention as well as improved outcomes when used with other cancer therapies.

Metformin and the more potent related compound Phenformin activate AMPK in cells, suggesting that Phenformin or other activators of AMPK might also be used as an adjunct to cancer therapy. Optimal use of these drugs will require a better understanding of cancer cell metabolism and identification of the signaling pathways that represent an Achilles' heel for cell proliferation or survival.

Metabolic tissues in mammals transform ingested food into a near-constant supply of glucose, glutamine, and lipids to balance the metabolic needs of both differentiated and proliferating tissues. Alterations in the appropriate balance of fuels and/or signal transduction pathways that deal with nutrient utilization may underlie the cancer predisposition associated with metabolic diseases such as diabetes and obesity.

A better understanding of how whole-body metabolism interacts with tumor metabolism may better define these risks and identify potential points of therapeutic intervention. In addition, it is possible that the cachexia associated with many cancers is exacerbated by the excess nutrient consumption by the tumor, which would affect whole-body metabolic regulation.

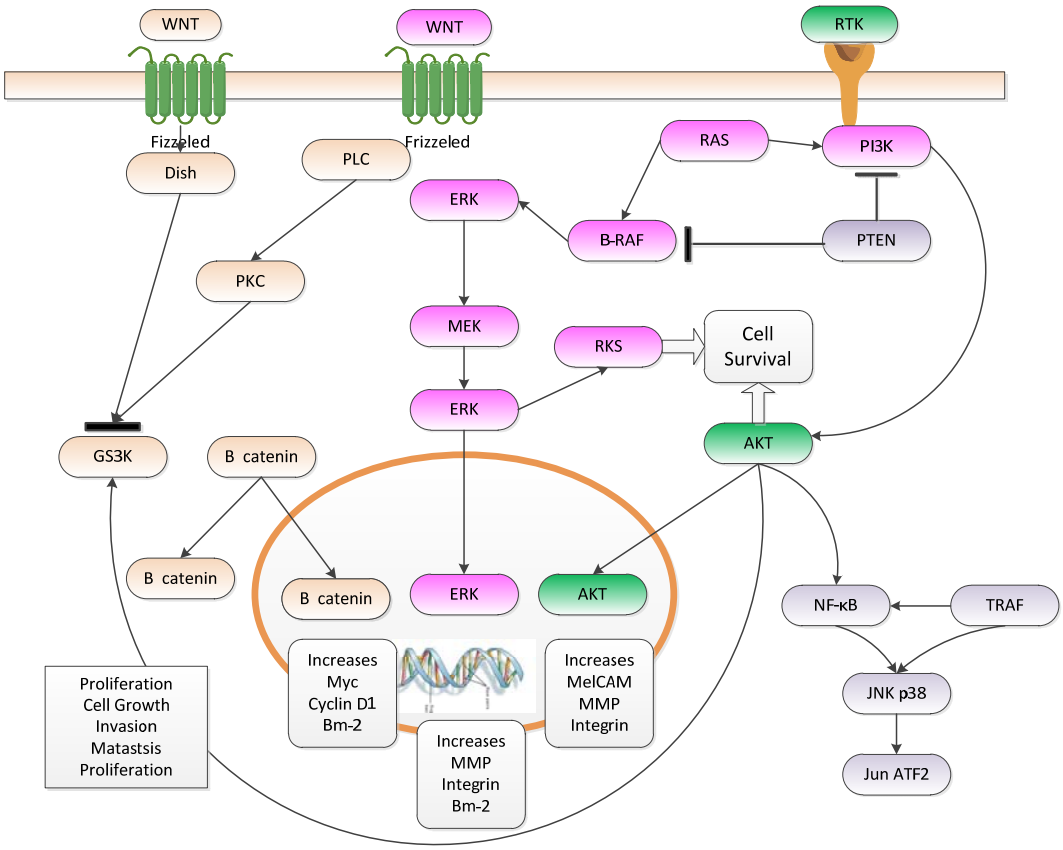
To this end, the potential role of dietary supplements and tight glucose control as adjuncts to cancer treatment is an active field of investigation.

The last comments has significant merit. The issue of glucose control, the impact of insulin related ligands and the pathway activation, all demonstrate this effect. One may then conjecture that when cancer cells migrate, they may do so along lines of higher glucose concentration. Thus the preference of hematological migration. Again the Warburg Hypothesis is less of interest in the classic sense than in the ability to better understand migratory and diffusional behavior.

2.3 PATHWAYS

Within the past few years the focus has changed somewhat to understanding pathways and seeking out mechanisms which can control, modify, or block these pathways. Pathways are essential control circuits generally within cells. They are the mechanisms within the cells which control the elements we have shown above. Pathways as we shall examine them are the control of proteins which are specific gene products which bring signals from the outside of the cell to the nucleus and in turn control the expression of the DNA.

The following is a typical set of such pathways found in melanoma cells[4]. We show several key types of players. Receptors such as Frizzled, external molecule like Wnt that bind and activate the receptors, cytoplasm proteins which interact with one another in the pathways and nuclear proteins used to control DNA actions and ultimately the DNA itself.



We will be analyzing many of these pathways as we progress. Simply what we observe is the outside of the cell reflected by receptors which in turn activate or deactivate certain proteins in the cytoplasm which in turn control proteins which control the nuclear DNA or independently control cell growth and proliferation. The above diagram reflects current knowledge as to the functioning of the melanocyte. Further details are available for each path. The questions we will address are:

[4] Hearing and Leong p 121.

1. What aberrations cause loss of cell growth management? Namely under normal conditions the above network works well and the cells are in a state of homeostasis. What gets broken and then how does it get broken? The current approach is to examine malignant cells and examine them for aberrant proteins such as B-RAF. It has been found that a certain B-RAF is found in 40-60% of all malignant melanomas. Yet the questions remain: how did it get that way and its aberrant behavior can be controlled is that all that has to be done?

2. What causes the aberrations? Is it always a gene malfunction and if so what causes the gene malfunction? In melanoma the UV A and B often are the most significant obvious cause but there are contradictions to that. First some people get melanoma in areas which never see the sun such as the colon or the mouth. Second areas of people which get considerable sun such as the face show lesser incidence than areas which get intermittent sun such as the arms and legs. Thus UV may be contributory but not the sole cause. One need look no further than X-rays and especially the backscatter x-rays.

3. Cancer cells seem to have a highly adaptive feature which allows them to change as threats to their progression are confronted. B-RAF blockers and the CML kinase inhibitor imatinib work just so long. What is the nature of that adaptive change. It appears as part of the basic system dynamics rather than some random event that just happens. Can we determine the fault lines from a systems model?

2.3.1 Melanoma Genes

We report a genome-wide association study for melanoma that was conducted by the GenoMEL Consortium. Our discovery phase included 2,981 individuals with melanoma and 1,982 study-specific control individuals of European ancestry, as well as an additional 6,426 control subjects from French or British populations, all of whom were genotyped for 317,000 or 610,000 single-nucleotide polymorphisms (SNPs). Our analysis replicated previously known melanoma susceptibility loci. Seven new regions with at least one SNP with $P < 10^{-5}$ and further local imputed or genotyped support were selected for replication using two other genome-wide studies (from Australia and Texas, USA). Additional replication came from case-control series from the UK and The Netherlands.

Variants at three of the seven loci replicated at $P < 10^{-3}$:

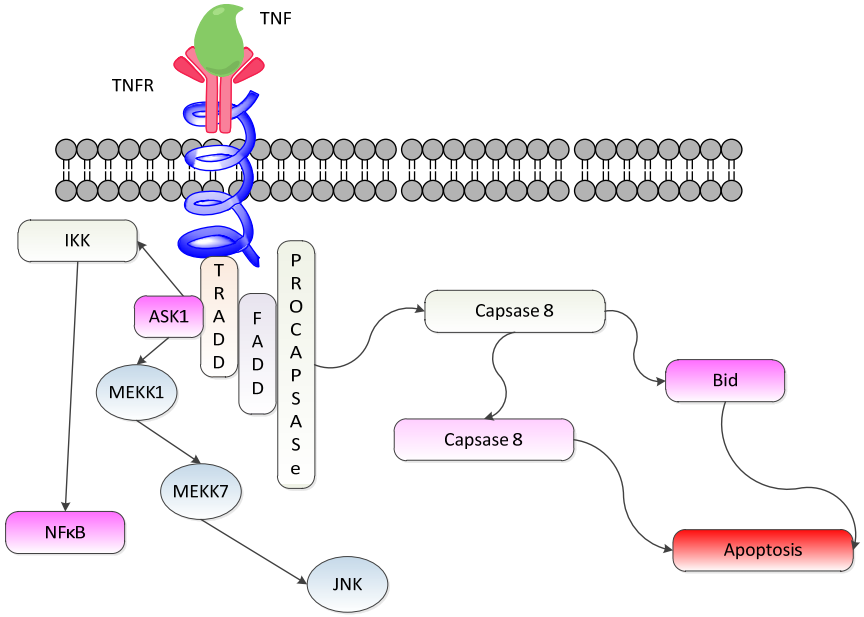
an SNP in ATM (rs1801516, overall $P = 3.4 \times 10^{-9}$),

an SNP in MX2 (rs45430, $P = 2.9 \times 10^{-9}$) and

an SNP adjacent to CASP8 (rs13016963, $P = 8.6 \times 10^{-10}$).

A fourth locus near *CCND1* remains of potential interest, showing suggestive but inconclusive evidence of replication (rs1485993, overall $P = 4.6 \times 10^{-7}$ under a fixed-effects model and $P = 1.2 \times 10^{-3}$ under a random-effects model).

These newly associated variants showed no association with nevus or pigmentation phenotypes in a large British case-control series[5]. Barrett et al, Genome-wide association study identifies three new melanoma susceptibility loci as shown below followed by their comments;



Caspase 8 *is a caspase protein, encoded by the CASP8 gene. It most likely acts upon caspase 3. CASP8 orthologs [1] have been identified in numerous mammals for which complete genome data are available. Unique orthologs are also present in birds.*

The CASP8 gene encodes a member of the cysteine-aspartic acid protease (caspase) family. Sequential activation of caspases plays a central role in the execution-phase of cell apoptosis. Caspases exist as inactive proenzymes composed of a prodomain, a large protease subunit, and a small protease subunit. Activation of caspases requires proteolytic processing at conserved internal aspartic residues to generate a heterodimeric enzyme consisting of the large and small subunits. This protein is involved in the programmed cell death induced by Fas and various apoptotic stimuli. The N-terminal FADD-like death effector domain of this protein suggests that it may interact with Fas-interacting protein FADD. This protein was detected in the insoluble fraction of the affected brain region from Huntington disease patients but not in those from normal controls, which implicated the role in neurodegenerative diseases. Many alternatively spliced transcript variants encoding different isoforms have been described, although not all variants have had their full-length sequences determined

Interferon-induced GTP-binding protein Mx2 is a protein that in humans is encoded by the *MX2* gene.[1][2][3]

[5] http://www.nature.com/ng/journal/vaop/ncurrent/pdf/ng.959.pdf

The protein encoded by this gene has a nuclear and a cytoplasmic form and is a member of both the dynamin family and the family of large GTPases. The nuclear form is localized in a granular pattern in the heterochromatin region beneath the nuclear envelope. A nuclear localization signal (NLS) is present at the amino terminal end of the nuclear form but is lacking in the cytoplasmic form due to use of an alternate translation start codon. This protein is upregulated by interferon-alpha but does not contain the antiviral activity of a similar myxovirus resistance protein 1
ATM

Ataxia telangiectasia mutated (ATM) is a serine/threonine protein kinase (EC 2.7.11.1) that is recruited and activated by DNA double-strand breaks. It phosphorylates several key proteins that initiate activation of the DNA damage checkpoint, leading to cell cycle arrest, DNA repair or apoptosis. Several of these targets, including p53, CHK2 and H2AX are tumor suppressors. The protein is named for the disorder Ataxia telangiectasia caused by mutations of ATM.

Ataxia telangiectasia (AT) is a rare human disease characterized by extreme cellular sensitivity to radiation and a predisposition to cancer. All AT patients contain mutations in the AT-mutated gene (ATM). Most other AT-like disorders are defective in genes encoding the MRN protein complex. One feature of the ATM protein is its rapid increase in kinase activity immediately following double-strand break formation.

The phenotypic manifestation of AT is due to the broad range of substrates for the ATM kinase, involving DNA repair, apoptosis, G_1/S, intra-S checkpoint and G_2/M checkpoints, gene regulation, translation initiation, and telomere maintenance.

Therefore a defect in ATM has severe consequences in repairing certain types of damage to DNA, and cancer may result from improper repair. AT patients have an increased risk for breast cancer that has been ascribed to ATM's interaction and phosphorylation of BRCA1 and its associated proteins following DNA damage. Certain kinds of leukemias and lymphomas, including Mantle cell lymphoma, T-ALL, atypical B cell chronic lymphocytic leukemia, and T-PLL are also associated with ATM defects.

Previous research by these and other scientists identified five pigmentation genes and three 'mole formation' genes, linked to melanoma risk. But the scientists have now discovered three new risk genes not associated with pigmentation or moles.

Four per cent of the UK population, around 2.3m people, will carry two copies of all three gene faults (one copy inherited from each parent). The average risk of developing melanoma is about one in 60. This goes up to one in 46 if a person has both copies of all three gene faults.
Lead author, Professor Tim Bishop, based in the Cancer Research UK centre at the University of Leeds, said: "We know that overexposure to UV increases the risk of developing melanoma – but this evidence shows that there are new additional genetic faults which can push up the risk further.

"It is fascinating to discover these new melanoma risk factors – and we expect that the results of similar studies underway will reveal even more."

Dr Lesley Walker, Cancer Research UK's director of cancer information, said: "These intriguing results provide deeper understanding of the causes of melanoma and provide a potential new approach to identify people most at risk of developing melanoma and other cancers."
One DNA fault was found in the region of a gene called MX2 linked to narcolepsy – a disease thought to be triggered by the immune system which causes people to fall asleep spontaneously.

Another fault was found in a gene called ATM involved in DNA repair – preventing cancer-causing mistakes being passed onto daughter cells. The third gene fault was found in the CASP8 gene, which plays a role in controlling cell spread by triggering automatic cell death.

"Melanoma is very highly mutated and is in some cases mutated approximately on an order of magnitude higher than other cancer types," said Yardena Samuels, Ph.D., an investigator in the Cancer Genetics Branch of the National Human Genome Research Institute's (NHGRI) Division of Intramural Research[6]. Dr. Samuels is part of a National Institutes of Health (NIH)-led team studying the genetics of melanoma and is the senior author of an article in the Sept. 25, 2011, early online issue of Nature Genetics that found that mutations in the metabotropic glutamate receptor-3 (GRM3) gene cause some cases of melanoma.

This newest melanoma-causing mutation joins a growing list of culprits. In April 2011, *Nature Genetics* published the team's first systematic genomic probe of melanoma. Using whole-exome sequencing, a technique that deciphers just the portions of the genome that code for proteins, the researchers identified 15 new mutations that drive cancer development and one that had previously been detected. The researchers found that one of the mutated genes, *GRIN2A*, is mutated in 25 percent of melanoma cases. It is located in the signaling pathway for the nerve cell messenger glutamate — the same pathway as *GRM3*.

In the current study, the researchers focused on mutations in the largest human gene family, G protein-coupled receptors (GPCRs). The significance of the receptors coded by GPCR genes is underscored by the fact that they are the targets of more than half of drugs approved by the U.S. Food and Drug Administration (FDA). Additionally, genes in the GPCR family regulate signal pathways for cell growth, the hallmark cellular activity in cancer.

NHGRI researchers and a colleague from the Johns Hopkins Sidney Kimmel Comprehensive Cancer Center in Baltimore designed and analyzed the new study, while National Cancer Institute (NCI) researchers, including Steven Rosenberg, M.D., Ph.D., chief of surgery at the NCI, and colleagues from the University of Texas MD Anderson Cancer Center in Houston and the University of Colorado Denver School of Medicine collected melanoma tumor samples.
The researchers obtained DNA from 11 melanoma tumor samples and sequenced the exon region — or protein-coding portion of the DNA sequence — that spanned the 734 GPCR genes.

Mutational analysis narrowed the interest of the researchers to a group of 11 genes in the GPCR family that contained two or more mutations. The team accessed an additional 80 melanoma samples and detected that one particular gene, *GRM3*, had a high mutation rate in the tumor samples. Furthermore, multiple tumor samples contained the precise mutation in the very same

[6] http://www.eurekalert.org/pub_releases/2011-10/uol-sdt100711.php and http://www.genome.gov/pfv.cfm?pageID=27545534

location within the gene, an occurrence characterized as a mutational 'hot spot.' This prevalent recurrence indicates that the mutation has a selective advantage, elevating its importance as a mutation that drives the development of cancer.

To explore the function of this gene, the researchers studied melanoma cells that harbor the mutations. In an experiment that heightened the function of mutated *GRM3*, the researchers detected increased activity of a signal pathway called the MAP kinase pathway, already known to be involved in melanoma. A kinase is a type of protein enzyme that modifies other proteins to cause some particular cell function. The pathway includes a kinase known as MEK targeted by current melanoma drugs. While some targeted treatments have been effective, tumors become drug resistant within months of treatment. The pathway also is associated with the most highly mutated gene in melanoma, called *BRAF*.

The researchers performed a test to detect whether the cells with the newly identified mutation in *GRM3* respond to the drug that inhibits the MEK pathway. They detected that mutated cells treated with a MEK inhibitor responded positively, dying off as they should rather than persistently replicating as cancer cells.

Further analysis showed that when the cells carry both a *BRAF* mutation and a *GRM3* mutation, the inhibitor compound selectively killed the cells that had the *GRM3* mutation. Dr. Samuels suggested that in some cases, failure of melanoma cells to respond to a MEK inhibitor might be due to those cells having a *BRAF* mutation but no *GRM3* mutation. Dr. Samuels predicts that prospective genetic analysis will enable differentiation of melanoma into subclasses.

"Melanoma has been subdivided by pathological characteristics," she said. "With the advent of in-depth genetic analyses, it may become possible to classify melanoma by its genetic alterations. Based on our two recent studies, I predict several melanoma subclasses will be identified in the near future."

Gene	*Location*	*Pathway*	*Controls*
BRAF	7q34	MAP/ERK	cell division, differentiation, and secretion
GRM3	7q21.1-q21.2	MEK	linked to the inhibition of the cyclic AMP cascade but differ in their agonist selectivities
GRIN2A	16p13.2		involved in long-term potentiation
ATM	11q22-q23		regulator of a wide variety of downstream proteins, including tumor suppressors: p53, BRCA1, CHK2, RAD17 and RAD9, DNA repair protein NBS1.
CASP8	2q33-q34		cell apoptosis
MX2	21q22.3		DNA repair
CCDN1	11q13		to interact with tumor suppressor protein Rb, alters cell cycle progression, are observed frequently in a variety of tumors and may contribute to tumorigenesis
CHK2	22q11; 22q12.1		cell cycle checkpoint regulator and putative tumor suppressor
H2AX	11q23.3		generates two transcripts through the use of the conserved stem-loop termination

BRAF: This gene encodes a protein belonging to the raf/mil family of serine/threonine protein kinases. This protein plays a role in regulating the MAP kinase/ERKs signaling pathway, which affects cell division, differentiation, and secretion. Mutations in this gene are associated with cardiofaciocutaneous syndrome, a disease characterized by heart defects, mental retardation and a distinctive facial appearance. Mutations in this gene have also been associated with various cancers, including non-Hodgkin lymphoma, colorectal cancer, malignant melanoma, thyroid carcinoma, non-small cell lung carcinoma, and adenocarcinoma of lung. A pseudogene, which is located on chromosome X, has been identified for this gene.[7]

GRM3: L-glutamate is the major excitatory neurotransmitter in the central nervous system and activates both ionotropic and metabotropic glutamate receptors. Glutamatergic neurotransmission is involved in most aspects of normal brain function and can be perturbed in many neuropathologic conditions. The metabotropic glutamate receptors are a family of G protein-coupled receptors, that have been divided into 3 groups on the basis of sequence homology, putative signal transduction mechanisms, and pharmacologic properties'[8]

[7] http://www.ncbi.nlm.nih.gov/gene/673

[8] http://www.ncbi.nlm.nih.gov/gene/2913

MX2: The protein encoded by this gene has a nuclear and a cytoplasmic form and is a member of both the dynamin family and the family of large GTPases. The nuclear form is localized in a granular pattern in the heterochromatin region beneath the nuclear envelope. A nuclear localization signal (NLS) is present at the amino terminal end of the nuclear form but is lacking in the cytoplasmic form due to use of an alternate translation start codon. This protein is upregulated by interferon-alpha but does not contain the antiviral activity of a similar myxovirus resistance protein 1.[9]

CASP8: This gene encodes a member of the cysteine-aspartic acid protease (caspase) family. Sequential activation of caspases plays a central role in the execution-phase of cell apoptosis. Caspases exist as inactive proenzymes composed of a prodomain, a large protease subunit, and a small protease subunit. Activation of caspases requires proteolytic processing at conserved internal aspartic residues to generate a heterodimeric enzyme consisting of the large and small subunits. This protein is involved in the programmed cell death induced by Fas and various apoptotic stimuli. The N-terminal FADD-like death effector domain of this protein suggests that it may interact with Fas-interacting protein FADD. This protein was detected in the insoluble fraction of the affected brain region from Huntington disease patients but not in those from normal controls, which implicated the role in neurodegenerative diseases. Many alternatively spliced transcript variants encoding different isoforms have been described, although not all variants have had their full-length sequences determined.[10]

ATM: The protein encoded by this gene belongs to the PI3/PI4-kinase family. This protein is an important cell cycle checkpoint kinase that phosphorylates; thus, it functions as a regulator of a wide variety of downstream proteins, including tumor suppressor proteins p53 and BRCA1, checkpoint kinase CHK2, checkpoint proteins RAD17 and RAD9, and DNA repair protein NBS1. This protein and the closely related kinase ATR are thought to be master controllers of cell cycle checkpoint signaling pathways that are required for cell response to DNA damage and for genome stability. Mutations in this gene are associated with ataxia telangiectasia, an autosomal recessive disorder.[11]

GRIN2A: N-methyl-D-aspartate (NMDA) receptors are a class of ionotropic glutamate-gated ion channels. These receptors have been shown to be involved in long-term potentiation, an activity-dependent increase in the efficiency of synaptic transmission thought to underlie certain kinds of memory and learning. NMDA receptor channels are heteromers composed of the key receptor subunit NMDAR1 (GRIN1) and 1 or more of the 4 NMDAR2 subunits: NMDAR2A (GRIN2A), NMDAR2B (GRIN2B), NMDAR2C (GRIN2C) and NMDAR2D (GRIN2D). Alternatively spliced transcript variants encoding different isoforms have been found for this gene.[12]

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[9] http://www.ncbi.nlm.nih.gov/gene/4600

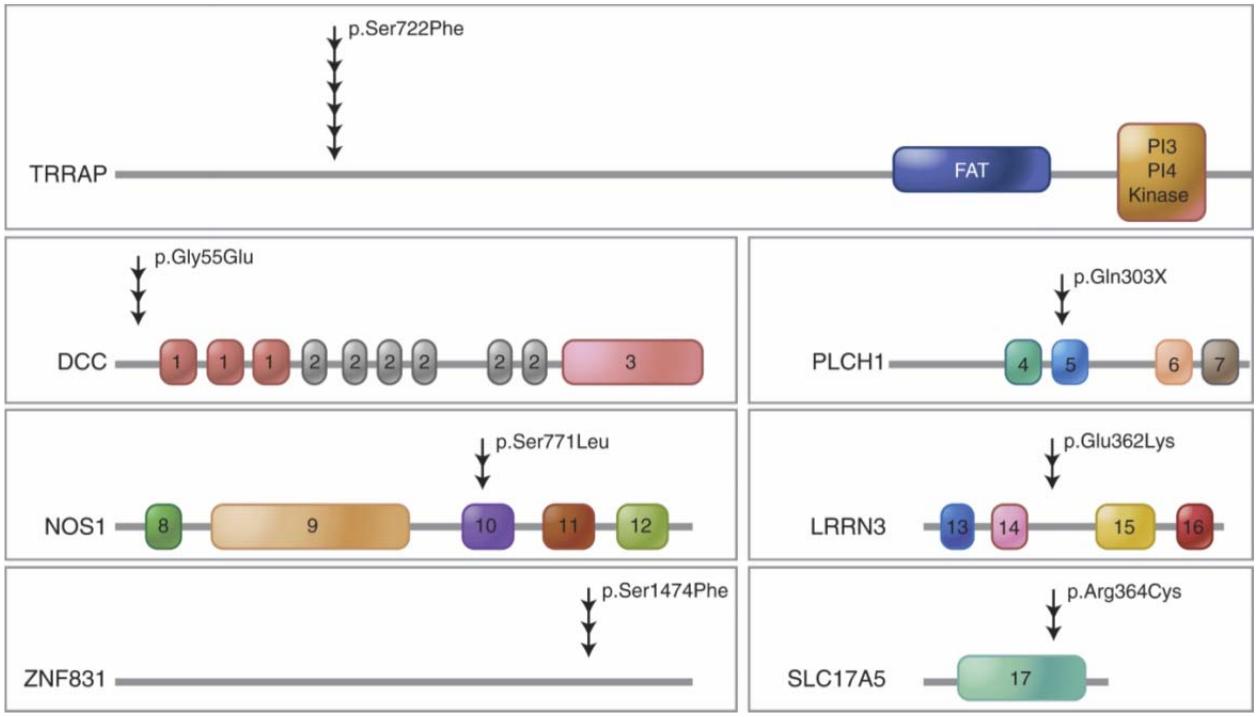
[10] http://www.ncbi.nlm.nih.gov/gene/841

[11] http://www.ncbi.nlm.nih.gov/gene/472

[12] http://www.ncbi.nlm.nih.gov/gene/2903

The incidence of melanoma is increasing more than any other cancer, and knowledge of its genetic alterations is limited. To systematically analyze such alterations, we performed whole-exome sequencing of 14 matched normal and metastatic tumor DNAs. Using stringent criteria, we identified 68 genes that appeared to be somatically mutated at elevated frequency, many of which are not known to be genetically altered in tumors.

Most importantly, we discovered that TRRAP harbored a recurrent mutation that clustered in one position (p. Ser722Phe) in 6 out of 167 affected individuals (~4%), as well as a previously unidentified gene, ***GRIN2A, which was mutated in 33% of melanoma samples.*** The nature, pattern and functional evaluation of the TRRAP recurrent mutation suggest that TRRAP functions as an oncogene. Our study provides, to our knowledge, the most comprehensive map of genetic alterations in melanoma to date and suggests that the glutamate signaling pathway is involved in this disease.[13]



CCND1: The protein encoded by this gene belongs to the highly conserved cyclin family, whose members are characterized by a dramatic periodicity in protein abundance throughout the cell cycle. Cyclins function as regulators of CDK kinases. Different cyclins exhibit distinct expression and degradation patterns which contribute to the temporal coordination of each mitotic event. This cyclin forms a complex with and functions as a regulatory subunit of CDK4 or CDK6, whose activity is required for cell cycle G1/S transition. This protein has been shown to interact with tumor suppressor protein Rb and the expression of this gene is regulated positively by Rb. Mutations, amplification and overexpression of this gene, which alters cell

[13] http://www.ncbi.nlm.nih.gov/pubmed/21499247/

cycle progression, are observed frequently in a variety of tumors and may contribute to tumorigenesis.[14]

CHK2: In response to DNA damage and replication blocks, cell cycle progression is halted through the control of critical cell cycle regulators. The protein encoded by this gene is a cell cycle checkpoint regulator and putative tumor suppressor. It contains a forkhead-associated protein interaction domain essential for activation in response to DNA damage and is rapidly phosphorylated in response to replication blocks and DNA damage. When activated, the encoded protein is known to inhibit CDC25C phosphatase, preventing entry into mitosis, and has been shown to stabilize the tumor suppressor protein p53, leading to cell cycle arrest in G1. In addition, this protein interacts with and phosphorylates BRCA1, allowing BRCA1 to restore survival after DNA damage. Mutations in this gene have been linked with Li-Fraumeni syndrome, a highly penetrant familial cancer phenotype usually associated with inherited mutations in TP53. Also, mutations in this gene are thought to confer a predisposition to sarcomas, breast cancer, and brain tumors. This nuclear protein is a member of the CDS1 subfamily of serine/threonine protein kinases. Three transcript variants encoding different isoforms have been found for this gene.[15]

H2AX: Histones are basic nuclear proteins that are responsible for the nucleosome structure of the chromosomal fiber in eukaryotes. Two molecules of each of the four core histones (H2A, H2B, H3, and H4) form an octamer, around which approximately 146 bp of DNA is wrapped in repeating units, called nucleosomes. The linker histone, H1, interacts with linker DNA between nucleosomes and functions in the compaction of chromatin into higher order structures. This gene encodes a member of the histone H2A family, and generates two transcripts through the use of the conserved stem-loop termination motif, and the polyA addition motif.[16]

2.3.2 Pathway Classification of Melanoma

There is an almost daily set of markers for a variety of cancers which are announced often with great fanfare. However the markers may or may not have any true meaning. We have discussed this in a prior posting and there we discussed the work by Venet et al as summarized by DeTours:

The signatures' prognostic potential can then be tested instantly in genome-wide compendia of expression profiles for hundreds of human tumors, all available for free in the public domain. Besides stem cells markers, signatures linked to all sorts of biological mechanisms or states have been shown to be associated with human cancer outcome. Indeed, several new signatures are published every month in prominent journals.

But such correlations are not all that they seem. The accumulation of signatures with all sorts of biological meaning, but nearly identical prognostic values, already looked suspicious to us and

[14] http://www.ncbi.nlm.nih.gov/gene/595

[15] http://www.ncbi.nlm.nih.gov/gene/11200

[16] http://www.ncbi.nlm.nih.gov/gene/3014

others back in 2007. It seemed that every newly discovered signature was prognostic. We collected from the literature some signatures with as little connection to cancer as possible. We found, for example, a signature of the blood cells of Japanese patients who were told jokes after lunch, and a signature derived from the microarray analysis of the brains from mice that suffered social defeat. Both of these signatures were associated with breast cancer outcome by any statistical standards.

Namely DeTours and his co-authors seem to say that it is all too easy to get markers for almost anything. In the context of Dougherty and his work, one must have an underlying verifiable model for the process and then from that verifiable model one can attempt to ascertain what elements may have failed. Then and only then can one obtain truly prognostic determinants which in turn may lead to means and methods to reduce the disease state.

For example in just the recent past we have papers which have identified the following for melanoma:

1. MAP2K1 and MAP2K2 mutations (Nature Genetics, 2011)
2. MAP3K5 and MAP3K9 mutations (Nature Genetics 2011)
3. ACP5 (Cancer Cell 2011)
4. The following complex (Cell Oct 2011):
    a. A Sleeping Beauty screen followed by MuTaME analysis discovered putative PTEN ceRNAs
    b. The PTEN ceRNA ZEB2 regulates PTEN in a miRNA-dependent manner
    c. ZEB2 loss activates PI3K/AKT signaling and promotes cell transformation
    d. Attenuated ZEB2 expression is found in melanoma and other human cancers
5. SNPs as reported at (Nature Genetics, 2011):
    a. an SNP in ATM
    b. an SNP in MX2 and
    c. an SNP adjacent to CASP8 .
    d. A fourth locus near CCND1 remains of potential interest,

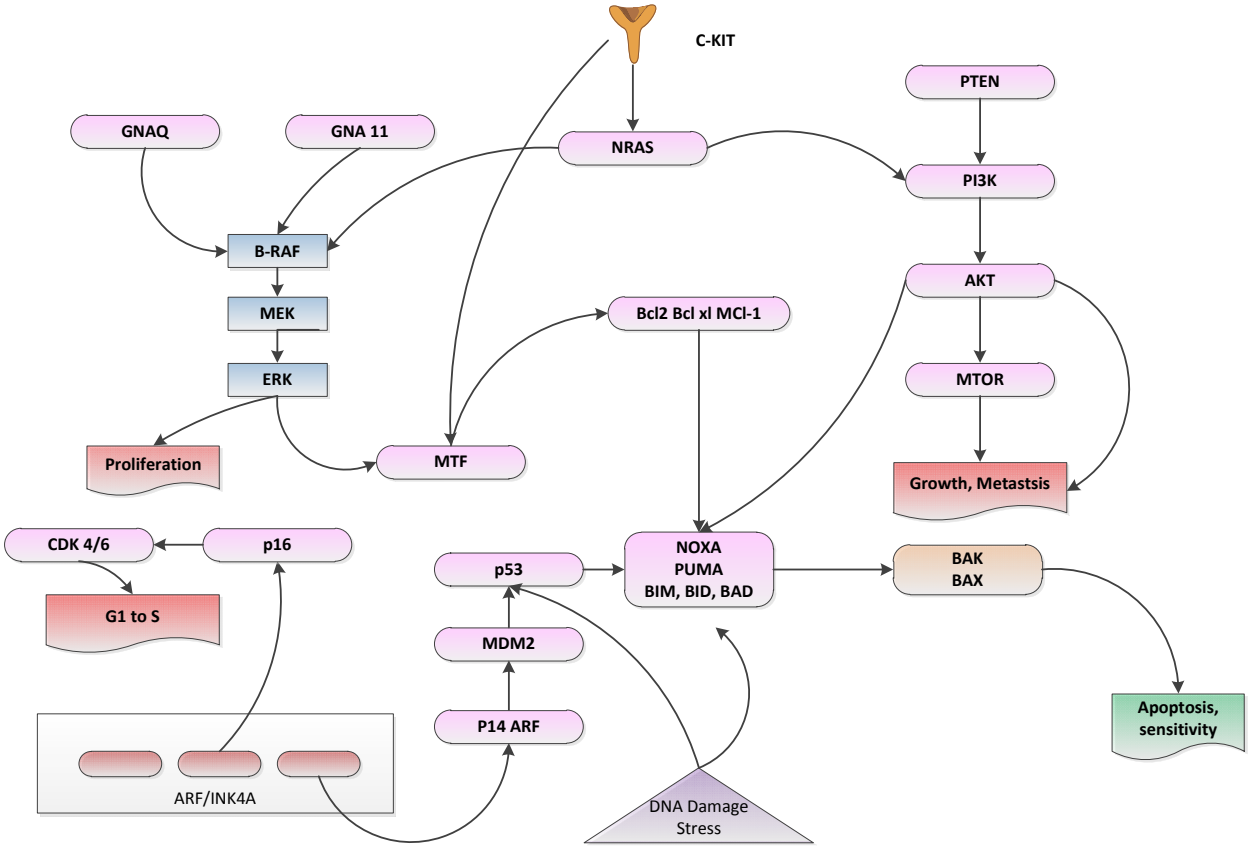
And the list goes on. As DeTours states, it may be all too easy to find aberrant genes, and even more so SNPs, independent of specific pathway models. And as I have argued, just within a pathway one may have a concern because it is also the intercellular signalling that is a concern as well. Even more so is the understanding of the process. Specifically:

1. A melanocyte may be normal until something happens. What is it that happens, does a SNP occur, why, when, and then what happens after that?
2. If a SNP occurs is that during the development of a DNA reading for protein generation or during cell replication. The opening of DNA for transcription may be the event which places

the melanocyte at risk. If so then what is the risk process. Could it be radiation as suspected, or is it the next step in a Vogelstein like progression. Namely there may have already been SNP damages and this one could be the final straw. Is it a micro RNA problem? The dynamics of this are essential.

3. Knowing pathways, is it possible to work backward and determine what the aberrant change or changes were? Pathway changes are reflected by their products.
4. What of the stem cell theory, must we look for the melanoma stem cell alone, and if so how can we identify it. The stem cell communicates, and that is a powerful mechanism to spread the cancer. How does it communicate and how is that related to the pathway.

Thus we look to understanding cancers in the context of pathways and then in the context of their intercellular pathways as well.



Understanding the pathway dynamics of melanoma has been progressing fairly well over the past few years. In a recent paper by Vidwans et al, the authors develop an interesting classification of melanoma based upon the specific pathway elements which may go awry. This is one of the first such classifications which goes beyond the classic morphological approach and even those using cell surface markers, This methods now looks at the cell dynamics and examines the malignancy based upon what specific pathway elements have failed. We show the pathway model used in the Vidwans paper above.

The now somewhat well understood B-RAF mutation, namely the V600E and discussed in the paper by Chapman et al:

Approximately 40 to 60% of cutaneous melanomas carry mutations in BRAF that lead to constitutive activation of downstream signaling through the MAPK pathway.[10,11] Approximately 90% of these mutations result in the substitution of glutamic acid for valine at codon 600 (BRAF V600E), although other activating mutations are known (e.g., BRAF V600K and BRAF V600R).

As Chapman et al state they have an inhibitor of the mutated B-RAF as follows:

Vemurafenib (PLX4032) is a potent inhibitor of mutated BRAF. It has marked antitumor effects against melanoma cell lines with the BRAF V600E mutation but not against cells with wild-type BRAF. A phase 1 trial established the maximum tolerated dose to be 960 mg twice daily and showed frequent tumor responses. A phase 2 trial involving patients who had received previous treatment for melanoma with the BRAF V600E mutation showed a confirmed response rate of 53%, with a median duration of response of 6.7 months.[16] We conducted a randomized phase 3 trial to determine whether vemurafenib would prolong the rate of overall or progression-free survival, as compared with dacarbazine.

As Bankhead states:

Patients with metastatic melanoma had an "astounding" 63% reduction in the risk of death when treated with an investigational agent that targets a mutation found in about half of the tumors, data from a large international trial showed.

Treatment with the BRAF inhibitor vemurafenib improved progression-free survival (PFS) by 74%. Analysis of six-month overall survival (OS) showed a 20% absolute difference between patients treated with vemurafenib versus dacarbazine.

Though follow-up is brief, the results already make a case for vemurafenib as the comparator for future trials of new agents for advanced melanoma, Paul. B. Chapman, MD, of Memorial Sloan-Kettering Cancer Center in New York City, said at the American Society of Clinical Oncology meeting.

"The median follow-up was only three months, yet the hazard ratio for death was 0.37 in favor of vemurafenib," Chapman said in an interview with MedPage Today. "That's an astounding difference that is almost never seen in oncology trials."

From 40% to 60% of cutaneous melanomas have BRAF mutations that activate downstream signaling through the MAP kinase pathway. About 90% of the mutations involve a specific substitution at codon 600 (BRAF V600E), Chapman and co-authors wrote…

The above demonstrates how understanding pathways we can target pathway drugs to mitigate the progression of the disease. However progression free survival is of limited duration. The cancer cell finds alternative paths to mutate. Thus the question is does one target one path after another as they progress or try a multi mix cocktail in hopes of preventing the development of

any new paths. Is it possible, for example, to stop the transcription of melanocytes all together, and thus stop any and all expression so as to silence say all pathways.

In another piece Bankhead states the cost issues:

Vemurafenib has an estimated cost of $56,000 for a six-month course of therapy, and ipilimumab costs about $120,000 for four weeks of treatment. Both drugs also have potentially serious adverse effects. In approving ipilimumab, the FDA cautioned that the drug has been associated with severe adverse effects that have included "severe to fatal autoimmune reactions."

The problem is that although the results are highly favorable for the short term, approximately six months, the long term is still questionable. It may be like imatinib and CML, namely there is a change in the cancer stem cells allowing a work-around of the blockage. Thus the costs would be considerable. Also the use of multiple drugs may as in leukemias result in “cures”. However the above costs, which may be at $20,000 per month of life extended, are excessive. The quality of life extended may not be the best and the drug while providing a “benefit” has not truly changed the end state, namely death of the patient. It has merely delayed the inevitable.

The issue of drugs, pathways, and targeting a sustainable remission is more than likely the target. As one has seen in many childhood cancers the goal of a sustainable remission is achieveable with cocktails of drugs and perhaps such may be the case here as well. Vidwans et al refer to their web site () which provides a superb interactive asset for linking pathway elements, disease stage, trials and specific modalities for possible mitigation and control[17]. The Table below is a modified version of the Vidwans table taken from their paper.

[17] see http://mmdm.cancercommons.org/ml/index.php/A_Melanoma_Molecular_Disease_Model

Melanoma Subtype	*Pathway*	*Gene/Biomarker*	*Diagnostic Technique*	*Potential Theraputic*
1.1	MAPK	BRAF	Targeted Sequencing	BRAF Inhibitor
1.2-1.3	MAPK	BRAF/PTEN	Targeted Sequencing and IHC	BRAF Inhibitor AND PI3K, AKT, mTOR inhibitors
1.2-1.3	MAPK	BRAF/AKT	Targeted Sequencing and copy number	BRAF Inhibitor AND AKT, mTOR inhibitors
1.4	MAPK	BRAF/CDK4	Targeted Sequencing and copy number / CGH	BRAF Inhibitor AND CDK inhibitors
2.1	c-KIT	c-KIT	Targeted Sequencing	
3.1	GNAQ/GNA11	GNAQ	Targeted Sequencing	MEK Inhibitor
3.2	GNAQ/GNA11	GNA11	Targeted Sequencing	MEK Inhibitor
4.1	NRAS	NRAS	Targeted Sequencing	MAPK, PI3K Inhibitor
5.1	MITF	MITF	Copy Number	HDAC Inhibitor
6.1	AKT/PI3K	PTEN	IHC	PI3K Inhibitor AKT Inhibitor mTOR Inhibitor
6.2	AKT/PI3K	AKT	Copy Number	AKT Inhibitor mTOR Inhibitor
6.3	AKT/PI3K	PI3K	IHC	PI3K Inhibitor AKT Inhibitor mTOR Inhibitor
7.1	CDK	CDKN2A	Targeted Sequencing	CDK Inhibitor HDAC Inhibitor
7.2	CDK	CDK4	Copy Number/CGH	CDK Inhibitor
7.3	CDK	CCDN1/Cyclin D	Copy Number/CGH	HDAC Inhibitor
8.1	P53/BCL	Bcl-2	IHC	
8.2	P53/BCL	p53	Targeted Sequencing	
9	TBD			

Now in contrast we have seen, as previously indicated, many papers where we have been presented with prognostic markers for melanoma and its development. Yet none seem to develop and verify them in the context of an underlying system model. The above mentioned work of Vidwans et al seems to be one of the first to commence that effort.

3 SKIN ANATOMY AND HISTOLOGY

The skin is the largest organ of the body. A skin cell is about 30 μm in diameter and the top layer of the skin, the epidermis, may be 5 to 15 cells thick and this 150 to 450 μm in thickness, about 0.5 mm at the deepest.

The dermatopathologist spends years of training to recognize and identify the multiplicity of skin disorders. This can be a very difficult process as exemplified by the ongoing debate on dysplastic nevi, are they pre-malignant or merely a condition unto themselves. One just needs to examine the years of work by Ackerman and colleagues in identifying melanocytic lesions. It is a complex and challenging process. It is a process which we will not even attempt to address herein.

However, our focus is on the specific intra and intercellular pathways. Examining the skin cellular structure provides a certain amount of insight and may be able to elucidate some of the extracellular flow issues including metastasis. However our goal in the chapter is not to provide details on dermatopathology but to provide a reasonable overview of its issues. Namely we want to be able to identify the melanocyte and to see what happens when a malignant condition is observed.

The challenge for the dermatopathologist in the coming years will be to not only use the visual clues, nor even the staining and immunohistological techniques, but to examine cells from a pathway distortion perspective both externally and internally. Namely do the cells express the desired or aberrant receptors and/or ligands and/or are the pathway elements normal or aberrant. Is the V600 BRAF present, for example? Currently there is a bifurcation of expertise in these areas and we may see an increase as time goes by and as we understand the pathway dynamics in a more complete manner.

The objectives of the Chapter are as follows:

1. Provide a general overview of the structure of the skin and the specific cells which compose this structure. The size of the cells and their “geographic” layout are essential to understanding the skin as an organ.

2. Provide a basic understanding of how the melanocyte integrates within that structure and what it appears to be in a health benign environment. This should provide a basis for understanding the importance of inter-cellular communications.

3. Identify the surrounding parts of the skin which interact with the melanocyte.

4. Identify abnormalities of the skin with emphasis on melanocytic disorders but also malignancies of other than melanocytes.

5. Identify and understand the simple melanocytic malignancy of melanoma and identifying and recognizing the typical morphological structures that are often found.

These objections, if met, will allow for a simple basis of understanding on how cells in a benign and malignant environment interact.

3.1 SKIN STRUCTURE AND MICROSCOPY

The skin is composed of multiple layers. Simply, there is the epidermis in the top layer and the dermis the bottom, with a basal layer in between.

In the top epidermal layer we have the following type of cells:

1. Keratinocytes (92+% of total)

These are the most abundant cells, which are always growing and migrating upward where they die off and fall off the top layer. The very top layers of the akin are the stratum corneum which is at the very outermost surface. Just below that layer is the stratum granulosom, the layer of dying keratinocytes.

2. Langerhans cells (4% of total)

They are dendritic in shape and are exclusively in the epidermis. They function as the macrophage in the epidermis by processing contact antigens which they present to specific T cells. The Langerhans cells are thus a part of the immune system. They also provide a transport and contact mechanism to the lymphatic system.

3. Merkel cells (<1% of total)

These cells are considered to be touch receptors and reside in the basal layer and are generally unseen in normal microscopic observation.

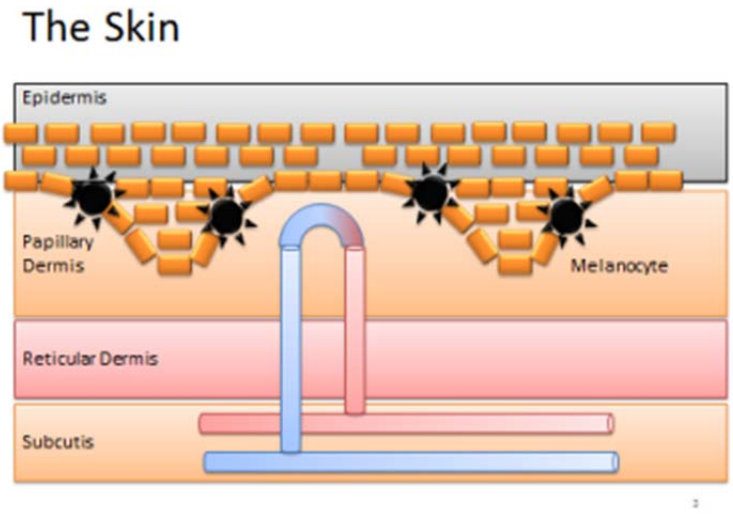
4. Melanocytes (3% of the total)

The melanocytes remain at the basal layer of the epidermis and have long tentacles which spread upward to the upper layers and from these tentacles they emit the melanocytes, the pigment of the skin and the general pigment of a nevus. Any movement, up or down, from the basal layer, of the melanocytes is pathognomonic of a malignancy of some form. Stability of the melanocyte is the *sine qua non* of a benign cell. Unlike the keratinocytes, which are reproducing and dying, the melanocytes are generally non-reproductive and stable. Their major function is to produce melanosomes. A single melanocyte provides about 30 keratinocytes with melanosomes.

The melanocytes are seen as clear cells in normal staining and appear as wedged between the keratinocytes.

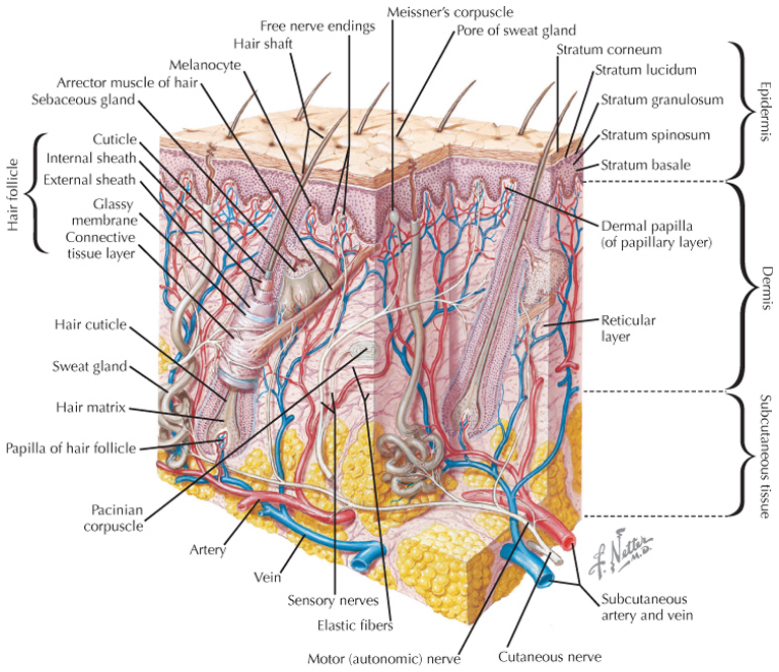
The figure below depicts the characteristics of the skin. The papillary dermis is about 0.4 to 0.6 mm in thickness and contains blood flow both from below and within the layer itself. It abuts the epidermis. It is composed of many collagen fibers and blood and never fibers. The blood flow to the basal layer and epidermal cells is via small capillaries that come up from the subcutis into

this top layer. The blood flow provides for oxygen and other nutrients and also eliminates any internal waste products and provides a pathway for the immune system.



The details from Netter are below. This shows a considerable amount of anatomical detail as compared to the above. Quoting Ovalle and Nahirney:

The skin *"... consists of **stratified squamous keratinized epithelium** on its outer part, called the **epidermis,** and an inner layer of **fibrous connective tissue,** called the **dermis.** A loose layer of **subcutaneous connective tissue,** the **hypodermis,** attaches skin to underlying structures and permits movement over most body parts. Skin has a dual embryologic origin: epidermis and its appendages derive mostly from surface ectoderm; dermis originates from mesoderm. The epidermis consists primarily of cells called keratinocytes, which make up more than 90% of the cell population. Other epidermal cells are **melanocytes** and Merkel cells, which derive from neural crest, and Langerhans cells, which have a monocytic origin. During embryonic development, skin appendages deriving from the epidermis grow down into the dermis*."



▲ **Schematic of skin and its appendages that shows the epidermis, dermis, and subcutaneous tissue.**

The skin is generally considered to be composed of the following layers. The first four are effectively in the epidermis and the last two in the dermis.

1. Stratum corneum: The top layer of the epidermis where the dead keratinocytes are lost.

2. Granular layer: Also called the Stratum Granulosum is below the top layer.

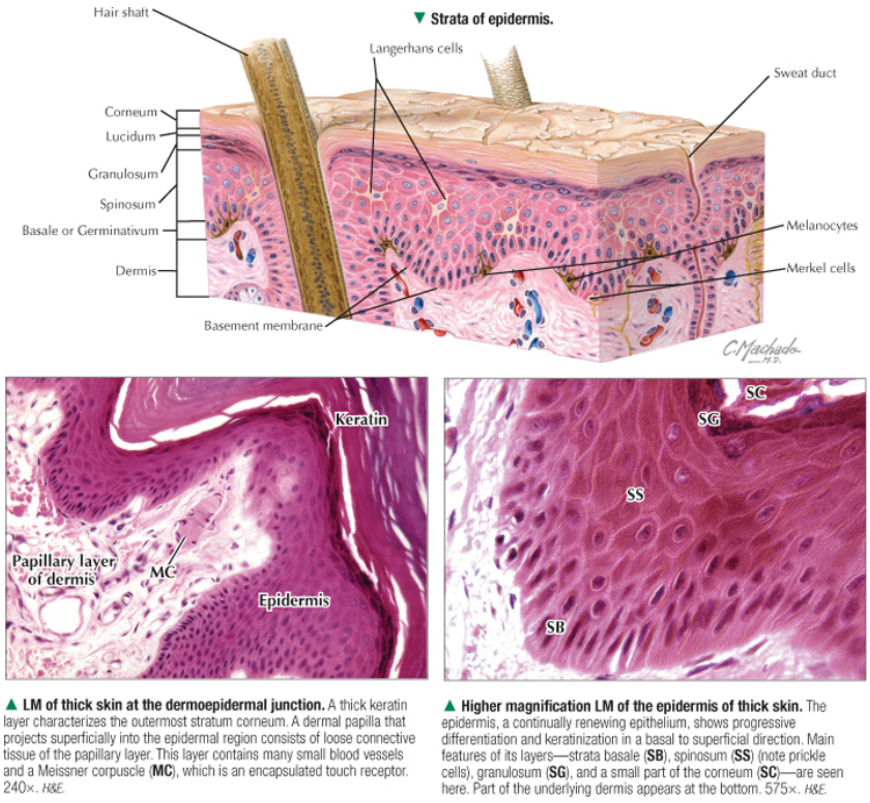
3. Spinous layer: Also called the Stratum Spinosum is just above the basal layer.

4. Basal Layer:

5. Papillary layer: Part of the dermis which is *composed of thin, haphazardly arranged collagen fibers.*

6. Reticular layer: Part of the dermis which is *the thicker lower layer and extends from the base of the papillary layer to the subcutaneous tissue and is composed of thick collagen fibers that are arranged parallel to the surface of the skin.*

From Ovalle and Nahirney we also have the graphic and specific cellular slides presenting actual views of the details:



▲ **LM of thick skin at the dermoepidermal junction.** A thick keratin layer characterizes the outermost stratum corneum. A dermal papilla that projects superficially into the epidermal region consists of loose connective tissue of the papillary layer. This layer contains many small blood vessels and a Meissner corpuscle (**MC**), which is an encapsulated touch receptor. 240×. *H&E.*

▲ **Higher magnification LM of the epidermis of thick skin.** The epidermis, a continually renewing epithelium, shows progressive differentiation and keratinization in a basal to superficial direction. Main features of its layers—strata basale (**SB**), spinosum (**SS**) (note prickle cells), granulosum (**SG**), and a small part of the corneum (**SC**)—are seen here. Part of the underlying dermis appears at the bottom. 575×. *H&E.*

The Figure above shows the detail of each of the respective layers.

We can now be more specific by each layer. Gartner and Hiatt have the following Table which gives detail on the cells from a histological perspective[18]:

[18] Gartner, L:., J. Hiatt, Color Textbook of Histology, Saunders (New York) 2007.

Layer	*Histological Features*
Epidermis	Derived from ectoderm; composed of stratified squamous keratinized epithelium (keratinocytes)
Stratum corneum	Numerous layers of dead flattened keratinized cells, keratinocytes, without nuclei and organelles (squames, or horny cells) that will be sloughed off.
Stratum lucidum*	Lightly stained thin layer of keratinocytes without nuclei and organelles; cells contain densely packed keratin filaments and eleidin.
Stratum granulosum*	A layer three to five cell layers thick; these keratinocytes still retain nuclei; cells contain large, coarse keratohyalin granules as well as membrane-coating granules.
Stratum spinosum	Thickest layer of epidermis, whose keratinocytes, known as prickle cells, interdigitate with one another by forming intercellular bridges and a large number of desmosomes; prickle cells have numerous tonofilaments and membrane-coating granules and are mitotically active; this layer also houses Langerhans cells.
Stratum basale (germinativum)	This single layer of cuboidal to low columnar, mitotically active cells is separated from the papillary layer of the dermis by a well-developed basement membrane; Merkel cells and melanocytes are also present in this layer.
Dermis	Derived from mesoderm; composed mostly of type I collagen and elastic fibers, the dermis is subdivided into two regions: the papillary layer and the reticular layer, a dense, irregular collagenous connective tissue.
Papillary layer	Interdigitates with epidermis, forming the dermal papilla component of the rete apparatus; type III collagen and elastic fibers in loose arrangement and anchoring fibrils (type VII collagen); abundant capillary beds, connective tissue cells, and mechanoreceptors are located in this layer; occasionally, melanocytes are also present in the papillary layer.
Reticular layer	Deepest layer of skin; type I collagen, thick elastic fibers, and connective tissue cells; contains sweat glands and their ducts, hair follicles and arrector pili muscles, and sebaceous glands as well as mechanoreceptors (such as pacinian corpuscles).

Let us now examine each layer more specifically. Our objective is first to understand what a normal skin looks like and then a malignancy.

3.1.1 Epidermis

The epidermis is the top layer of the skin.

It is composed of the following is composed of the following four layers (deep to superficial or from the bottom to the surface)[19]:

1. Basal layer: source of replacement cells and epidermal stem cells. The cells are generated here and the keratinocytes then migrate upward. Also the melanocytes are located here and send their tentacles and melanosomes upward from this layer. Normally melanocytes do not move from this point.

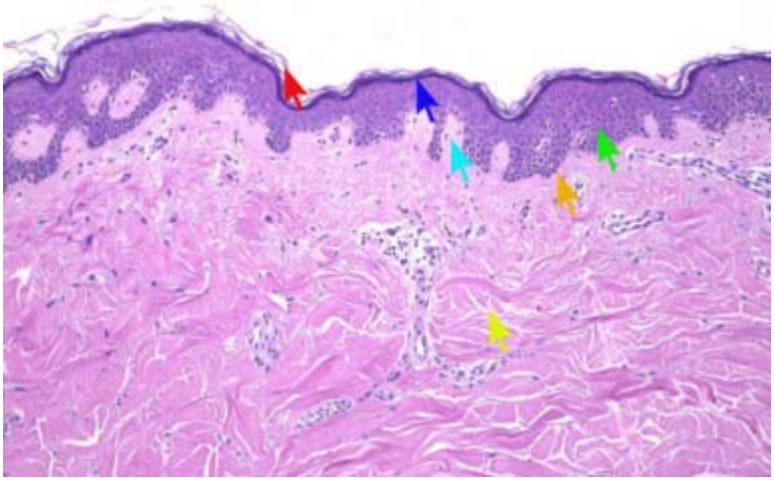
2. Spinous layer: center of epidermis that has a spiny appearance due to desmosomal junctions, area where keratinocytes produce keratin;

[19] http://missinglink.ucsf.edu/lm/DermatologyGlossary/index.html

3. Granular cell layer: site of the epidermis' water barrier;

4. Stratum corneum: thick outer layers of flattened keratinized non-nucleated cells that provide a barrier against trauma and infection. This layer shows dying or deal keratinocytes flaking off.

The details are below. Here we have the epidermis, composed of keratinocytes and the basal layer where we have melanocytes. The melanocytes have tentacles which grow through the keratinocyte layer and exude melanosomes, the dark pigmentation we see in colored skin patches. Below are the dermis and then the subcutis. The blood flow goes from arteries to veins and provides nutrients to the adjacent layers. A nevus will be seen as an agglomeration of melanocytes at the basal layer.



Habif defines the epidermis using a slightly different level of detail:

The epidermis is the outermost part of the skin; it is stratified squamous epithelium. The thickness of the epidermis ranges from 0.05 mm on the eyelids to 1.5 mm on the palms and soles. The microscopic anatomy of the epidermal-dermal junction is complex….

The innermost layer of the epidermis consists of a single row of columnar cells called basal cells. Basal cells divide to form keratinocytes, which comprise the spinous layer.

The cells of the spinous layer are connected to each other by intercellular bridges or spines, which appear histologically as lines between cells.

The keratinocytes synthesize insoluble protein, which remains in the cell and eventually becomes a major component of the outer layer (the stratum and corneum). The cells continue to flatten, and their cytoplasm appears granular (stratum granulosum); they finally die as they reach the surface to form the stratum corneum.

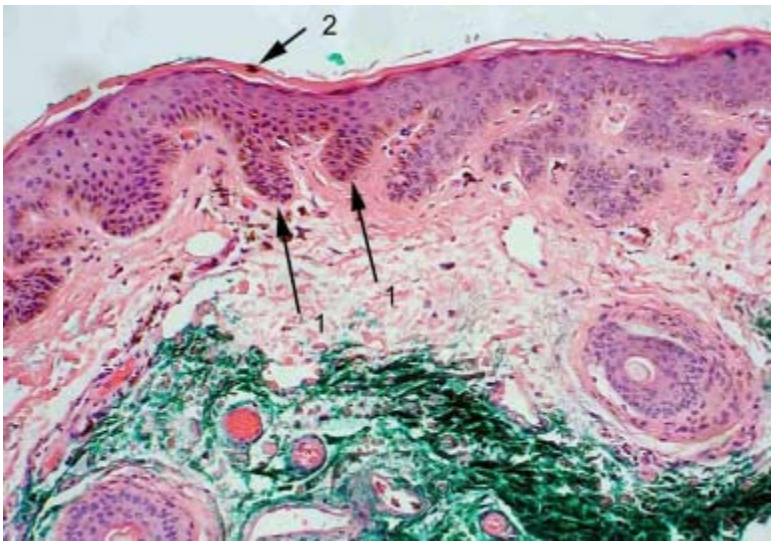
There are three types of branched cells in the epidermis: the melanocyte, which synthesizes pigment (melanin); the Langerhans cell, which serves as a frontline element in immune reactions of the skin; and the Merkel cell, the function of which is not clearly defined.

3.1.2 Dermis

In contrast the dermis is described as follows. The dermis is composed of cells, connective tissue, and ground substance and can contain blood and lymphatic vessels, nerves, glands, and hair follicles. It ranges from 1-4mm in thickness, making it much thicker than the epidermis. The dermis is divided into two layers: the papillary dermis and the reticular dermis. The papillary dermis is the region closest to the epidermis with papillae interdigitating with the epidermis; here, collagen fibers are thinner and loosely packed. In the deeper reticular dermis, collagen fibers are thicker and more densely and irregularly arranged. Elastin fibers are found in both the papillary and the reticular dermis, but they are more numerous within the latter.

Cutaneous appendages, like hair follicles, originate from the dermis. Blood vessels and nerves course through the dermis which supplies strength to the skin by its collagen and elastic fiber network. The vasculature to the skin is arranged in two plexi, the superficial plexus, located within the papillary dermis, and the deep plexus, located within the reticular dermis.

The basal layer is often seen as flowing in a wave like manner creating what are called Rete Ridges. We show them below:



Again quoting Habif we have:

The dermis varies in thickness from 0.3 mm on the eyelid to 3.0 mm on the back; it is composed of three types of connective tissue: collagen, elastic tissue, and reticular fibers.

*The dermis is divided into two layers: the thin upper layer, called the **papillary layer**, is composed of thin, haphazardly arranged collagen fibers; the thicker lower layer, called the*

***reticular layer**, extends from the base of the papillary layer to the subcutaneous tissue and is composed of thick collagen fibers that are arranged parallel to the surface of the skin.*

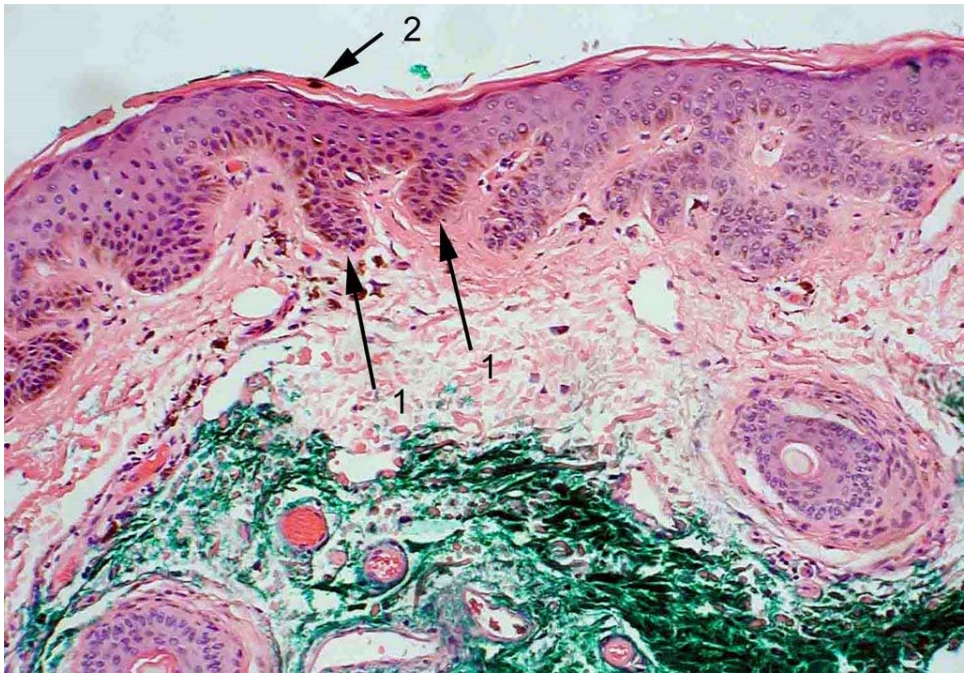
Histiocytes are wandering macrophages that accumulate hemosiderin, melanin, and debris created by inflammation. Mast cells, located primarily around blood vessels, manufacture and release histamine and heparin.

3.1.3 Some Basic Skin Architectural Elements

We now present some important specifics that will be incorporated in the description of melanoma.

3.1.3.1 Rete Ridges

Rete are a net or mesh of cells often at the bottom of the basal layer. As seen below they tend to extend downward and from time to time they may connect, anastamatose, but generally they are finger like extensions.



A microscopic description is as follows:

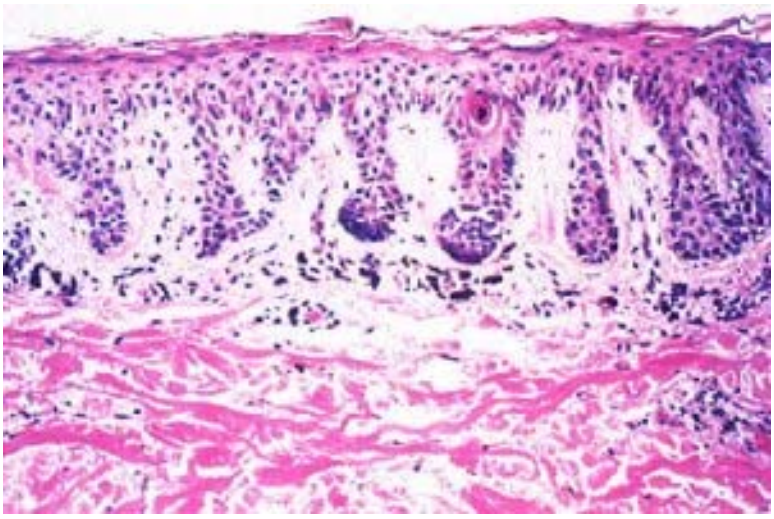
A. There are plump fibroblasts within the upper part of the dermis of this dome-shaped papule.

B. There is a proliferation of enlarged melanocytes arranged as solitary units and as nests within the epidermis, at the dermo-epidermal junction and down epithelial structures of adnexa. There is marked solar elastosis.

If this biopsy captured the entire pigmented lesion this could be a junctional melanocytic nevus

3.1.3.2 Lentigenes

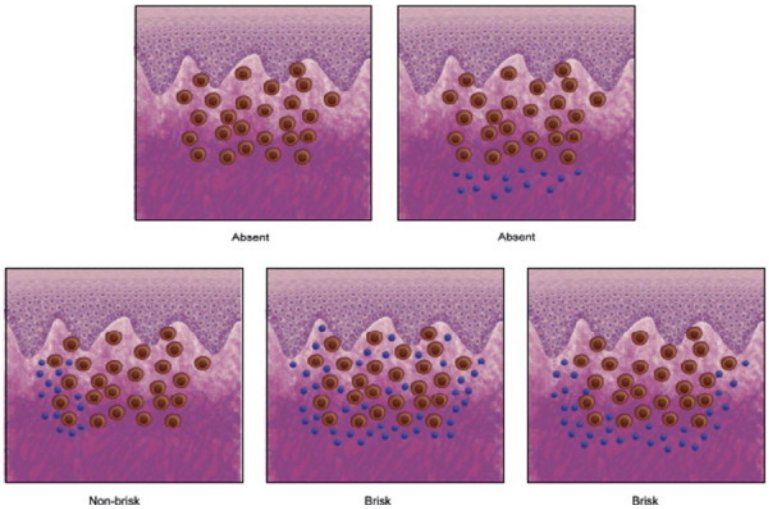
Lentigenes have a distinct histologic pattern of elongated, club-shaped rete ridges which often anastomose.



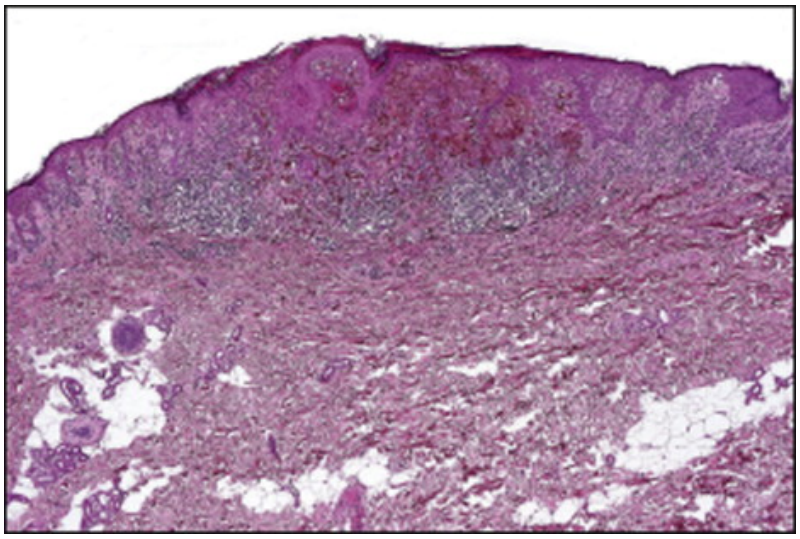
Lentigenes (which is the plural of lentigo) is a flat brownish spot on the skin resulting from excess melanin. Lentigo maligna is a macular (raised) patch. One can see the melanocytes in the basal layer as he clear cells.

3.1.3.3 Tumor Infiltrating Lymphocytes

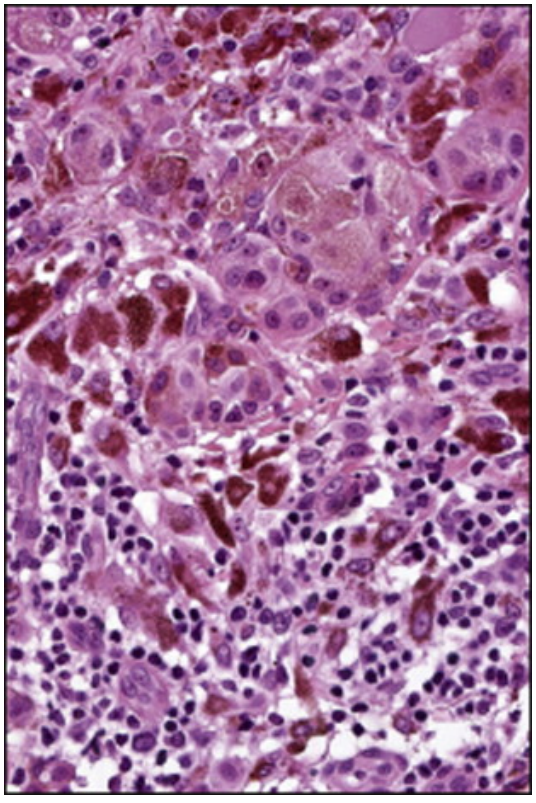
Tumor infiltrating lymphocytes are lymphocytes which have left the blood stream and have infiltrated the cellular area. Melanomas frequently have TILs which is a good prognostic factor. We show from McKee and Calonje the examples of TILs.



We show a cell below demonstrating TILs. They are the dark spotted areas.



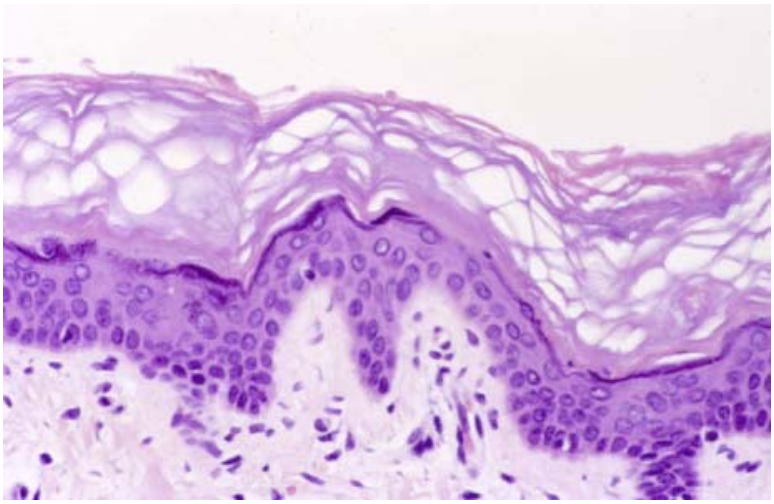
The following is the high power description.



3.1.3.4 Melanocytes

Melanoma is a cancer of the melanocyte. The normal condition of the melanocyte is to stay at the basal layer of the epidermis, with its dendritic arms moving upward and distributing melanosomes to the keratinocytes in response to ultra violet radiation. It is a protective function. The trouble starts when the melanocyte fails to stay put and to migrate and then to multiply.

We show a typical histological section below[20]:

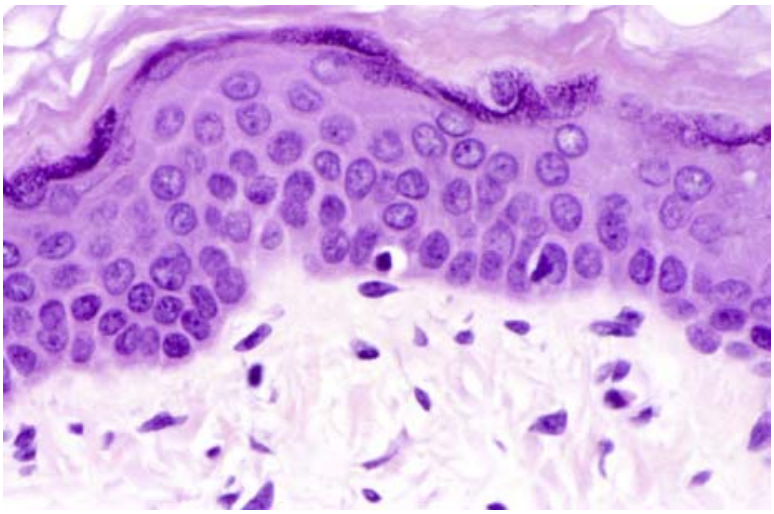


This shows the typical structure comparable to what we have shown above.

The following is an example showing a prominent melanocyte[21]. Note at the basal layer, namely the bottom layer, we see a dark nucleolus with a clear cytoplasm. That is a single melanocyte, and there is another to its right. The melanocytes normally have a clear appearance, are at the basal layer and often have a prominent nucleus.

[20] http://pathology.mc.duke.edu/research/Histo_course/epi1.jpg

[21] http://pathology.mc.duke.edu/research/Histo_course/melanocyte.jpg

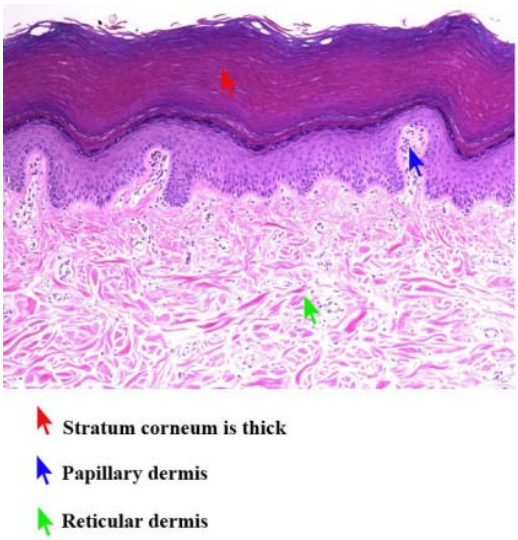


The above is a 200X magnification. One can see the keratinocytes building from the bottom of the basal layer and moving upward where they get squashed down and then slough off the surface. The melanocytes have a generally longer life cycle and they remain fixed.

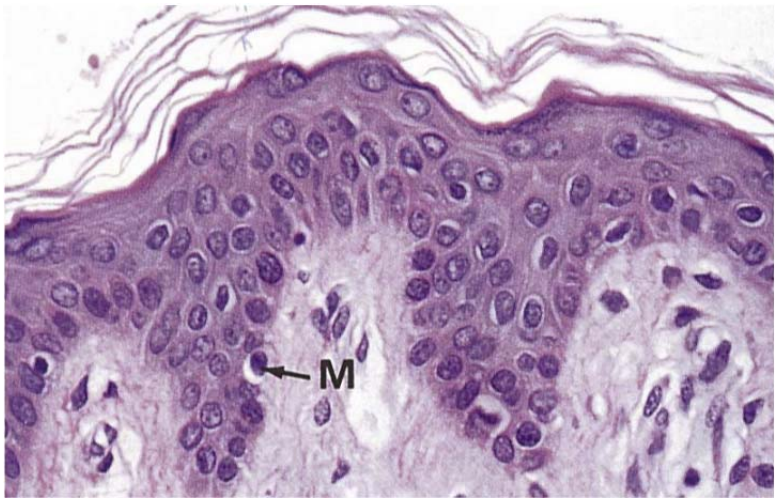
In this chapter we look at the histology of the melanocyte. In many ways it differs from other cancers in that we can focus on the aberrant behavior of a single cell type, the melanocyte. In a benign state it belongs to the basal layer and should remain there. The melanocyte produces melanosomes for pigmentation. When it loses its normal control mechanism and starts to become a pre-malignant cell the melanocytes lose their location sense. They move from the basal layer to the epidermis and then can be called melanoma in situ. Then they spread outward from the basal layer and become superficial spreading melanoma. Then they start the vertical stage and that is when we see metastatic potential. We examine those stages herein.

We continue to examine the normal skin histology. The following slide details more structure of the normal skin[22]. It demonstrates the dermis structure as well, specifically the papillary and reticular dermis. :

[22] http://missinglink.ucsf.edu/lm/DermatologyGlossary/normal_skin.html



The following shows another example of a melanocyte in normal skin[23]:



Again, in the above, we see the melanocyte in the basal layer, it has a clear cytoplasm and it has a prominent and round nucleolus.

3.2 SKIN CONDITIONS AND DISORDERS

Before examining melanoma we look at a few common skin conditions and disorders. We have considered Lentigo maligna in the previous section and it may also be considered as a disorder here as well. However we used it to emphasize the rete construct.

3.2.1 Nevi and Benign Pigmentation

[23] http://www.oucom.ohiou.edu/dbms-witmer/Downloads/Basic%20Skin%20Histology2-21-01.pdf

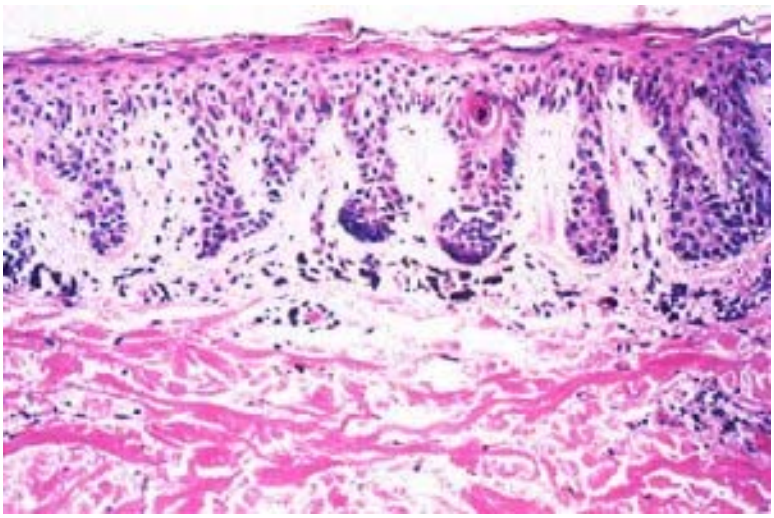
The microscopic appearance of a benign melanocytic nevus depends on the type of nevus and what type of skin it may be located on. In general, melanocytic nevi are symmetrical, well delineated and circumscribed growths of melanocytes. This growth can be only in the epidermis (junctional nevi), only in the dermis (intradermal nevi) or in both (compound nevi).
The melanocytes may have different cytologic appearances and arrangements depending on the type of benign nevus. A blue nevus is intradermal, roughly wedge-shaped and has thin, delicate melanocytes that may be in a dense fibrotic stroma with abundant melanin pigment.
A common intradermal or junctional nevus has round and polygonal melanocytes which are nested or grouped.

A Clark's nevus has a more prominent proliferation of melanocytes, many of which are arranged as nests along rete ridges. The rete ridges are often "bridged" (connected at their bases). The papillary dermis is classically fibrotic in Clark's nevi.

3.2.2 Actinic (Solar) Lentigo

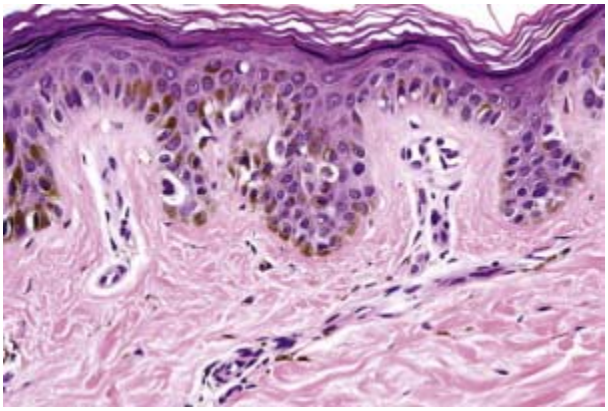
The differential diagnosis of lentiginous lesions can be challenging. It has been written that solar lentigo is the nascent lesion for seborrheric keratosis. Actinic or Solar lentigo generally features compact orthokerotosis with little melanin in the keratin layer (arrow 2), epidermal hyperplasia with bulbous rete ridges, pigmented keratinocytes especially prominent at the bulbs of the rete ridges (arrows 1).

Melanocytes are generally not found in the epidermis (the absence of melanocytic hyperplasia is a key feature in this entity). Mild chronic inflammation may be present but often without pigment laden macrophages in the dermis. Lamellar collagen around the rete pegs is absent. While key features exist, the lack of melanocytic hyperplasia, the presence of bulbous tips, but also in contrast to some writings, there are numerous pigmented melanophages that are perivascular.



Lentigenes have a distinct histologic pattern of elongated, club-shaped rete ridges which often anastomose (connect together or bridge across).

From Brinster et al (see Fig 7) we have below:



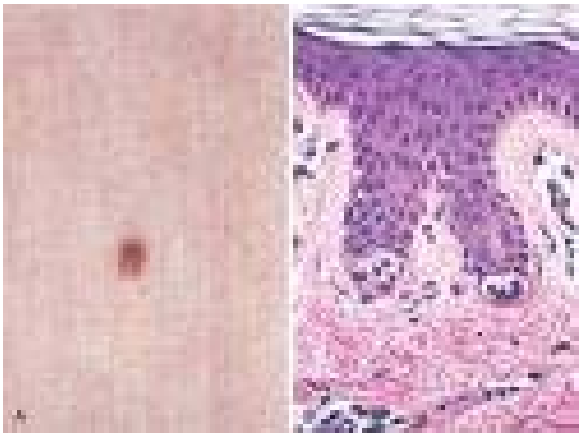
Actinic lentigo. Increased numbers of basally located melanocytes with a characteristic retraction artifact are present. There is no cytological atypia.

3.2.3 Junctional Nevus

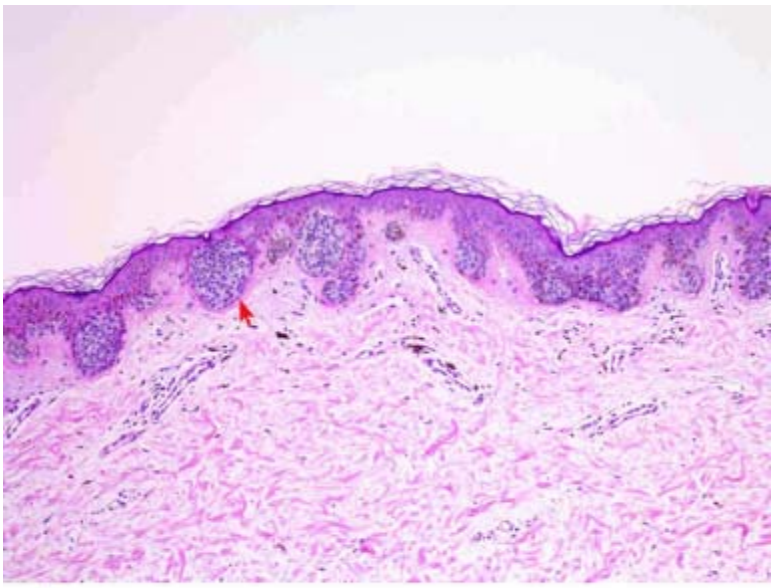
Nevocellular nevus, junctional type.

A, In clinical appearance, lesions are small, relatively flat, symmetric, and uniform.

B, On histologic examination, junctional nevi are characterized by rounded nests of nevus cells originating at the tips of rete ridges along the dermoepidermal junction.



Note below the clusters of melanocytes at the basal layer. The melanocytes are somewhat transparent with darkened nuclei. The clusters are uniform and somewhat well structured.



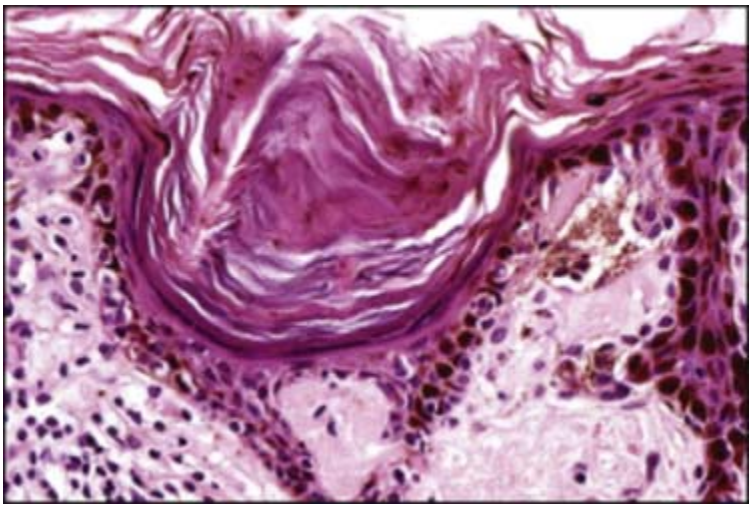
"nests" that are junctional (only in epidermis)

Specifically they are characterized as: (i) Relatively symmetrical, (ii) round nests of (iii) cohesive cells (iv) within lower part of dermis.

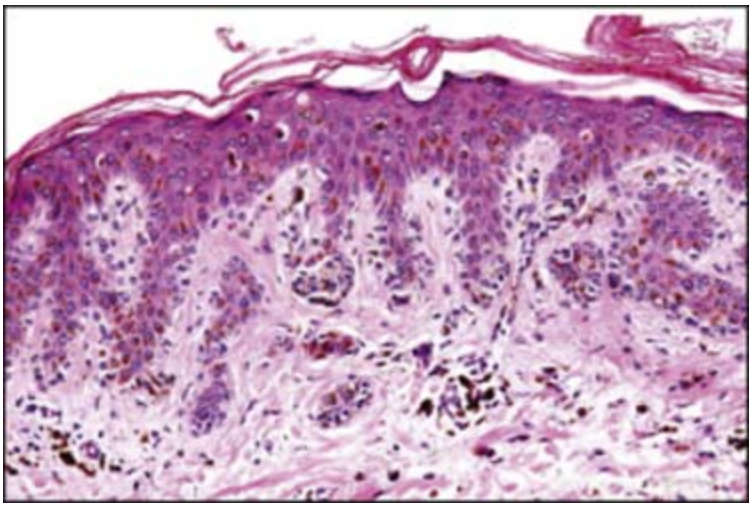
Characteristics of intradermal nevus cells, also type A nevus cells:

- Cuboidal, oval or spindle
- Cytoplasm gray often distinctly outlined, may exhibit some dendritic processes
- nucleus large round and oval
- nucleolus small
- melanin when present disposed in variably large clumps

The following is a junctional nevus, from McKee et al Fig 2.9, and the clusters of melanocytes are seen on the right hand side, with clear cytoplasm and darkened nucleus at the basal layer.



In McKee et al Fig 2.16 we have a more complicated nevus:



Note the clusters of melanocytes at the base of the rete ridges.

In melanocytic neoplasia we have the following general histological characteristics[24]:

1. Generally symmetrical and circumscribed, although a shoulder may sometimes be evident.

2. Proliferation of bland-appearing melanocytes in nests and cords.

3. Junctional nevus: intraepidermal proliferation of nested melanocytes along the dermal–epidermal junction, nests typically show rete-tip predominance, absence of pagetoid spread and cytological atypia.

4. Compound nevus: presence of nested melanocytes within both the epidermis and dermis, acanthosis, rarely pseudoepitheliomatous hyperplasia.

5. Dermal nevus: presence of nested melanocytes within the superficial dermis, nest formation typically lost with depth, nevus cells displaying an "infiltrating" growth pattern at base, sometimes diffuse distribution throughout the nevus.

6. Lentiginous melanocytic hyperplasia of single-cell melanocytes may be seen along the dermal–epidermal junction.

7. Maturation with depth: melanocytic nests and individual melanocytes diminish in size with descent into the dermis, nuclei become smaller, and pigmentation diminishes.

8. Type-A cells have abundant cytoplasm and vesicular nuclei with prominent nucleoli; type-B cells have less conspicuous cytoplasm and hyperchromatic nuclei; type-C cells have a

[24] See Brinster et al, Dermatopathology,

spindled morphology resembling Schwann cells and sometimes show pseudo-Meisnerian corpuscles (neurotization)

9. Multinucleate giant cells with senescent atypia common in dermal nevi.

10. Calcification of follicular remnants sometimes seen, rarely osteoid.

11. Signet-ring cell change very rare.

12. Fatty infiltration sometimes present

13. Pseudovascular change possibly resulting from injection of local anesthetic.

14. Lack, or only very rarely find, mitoses within the dermis; no atypical mitoses

3.2.4 Dysplastic Nevus

Dysplastic nevus is a class of nevi which are somewhat between a benign nevus and a melanoma. They have been posed as precursors of melanoma and they have been argued to be benign and a class of nevi. However there is a strong incidence of melanomas on patients with dysplastic nevi, especially a large number of such nevi.

The dysplastic nevus is histologically characterized by:

1. Junctional component extends beyond dermal component (shoulder).

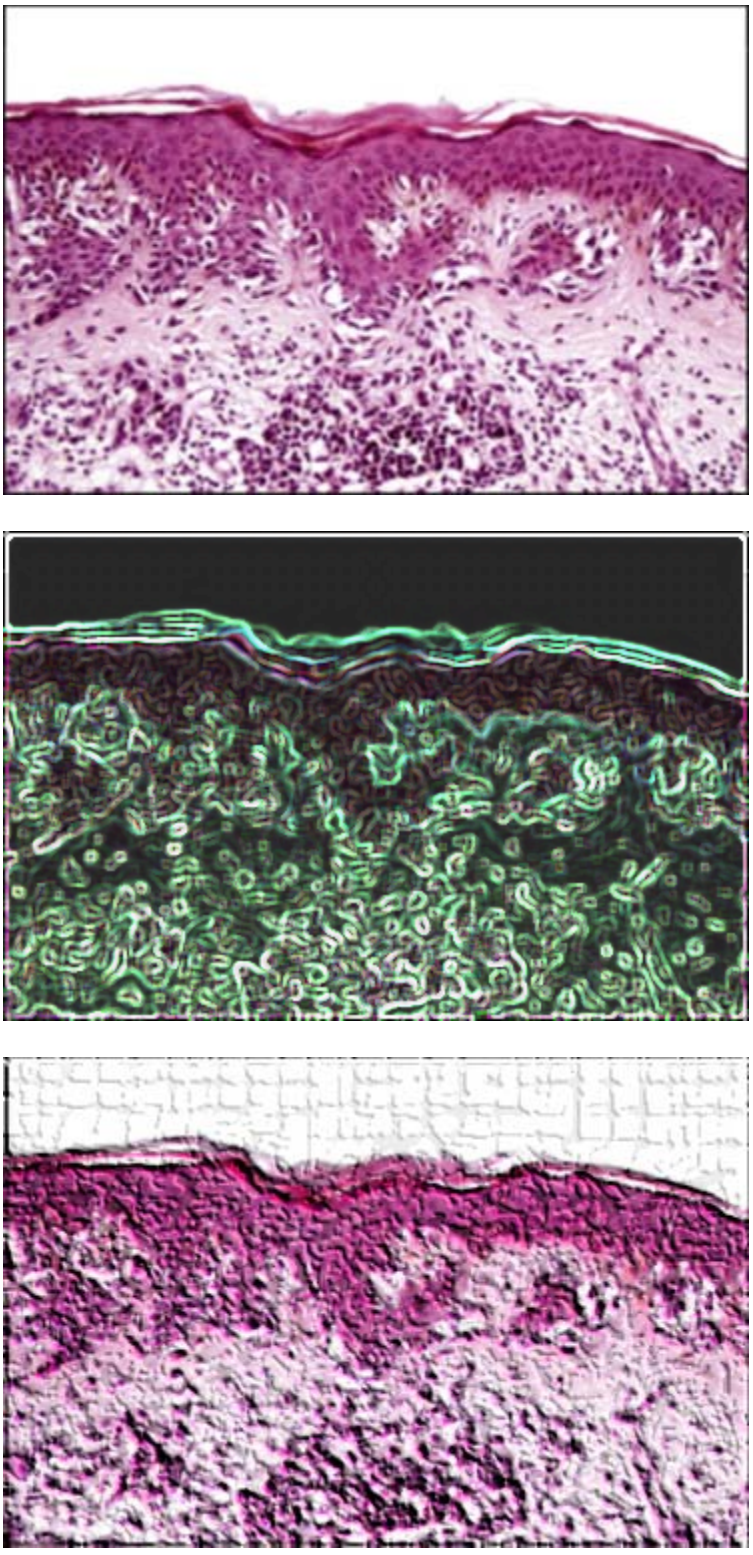
2. Disorganized and randomly distributed lentiginous and nested components (architectural disorder).

3. Absence of rete tip predominance.

4. Nests are often horizontally orientated.

5. Bridging between adjacent nests.

From McKee et al Fig 5.7 we have a high power view of a dysplastic nevus.



3.2.5 Melanoma in Situ

Melanoma in situ is (MIS) considered a localized but potentially malignant melanocyte growth. As we had indicated melanocytes are fixed in the basal layer and when Eosin stained the appear

somewhat transparent. In MIS they start to lose their fixation, and they start to have a shape which is larger than normal. We show this below. Also MIS is often found is sun damaged skin so there is also solar damage as well. The red arrow depicts a melanocyte demonstrating larger shape and the red arrow depicts the sun damage.

Now consider a typical pathology report, and notice the ambiguity between junctional nevus and MIS. A microscopic description read as follows (taken from a dermatopathology report):

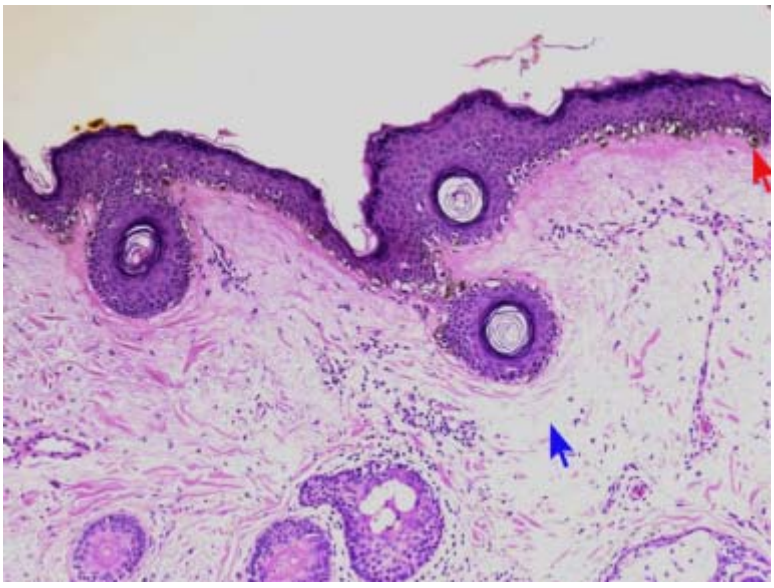
1. There are plump fibroblasts within the upper part of the dermis of this dome-shaped papule.

2. There is a proliferation of enlarged melanocytes arranged as solitary units and as nests within the epidermis, at the dermo-epidermal junction and down epithelial structures of adnexa.
There is marked solar elastosis.

LEFT FOREARM - ATYPICAL INTRAEPIDERMAL MELANOCYTIC PROLIFERATION.

Note: If this biopsy captured the entire pigmented lesion this could be a junctional melanocytic nevus.

Note there is considerable concern as to whether this is a MIS or a junctional nevus with some slightly abnormal melanocytic movements. The typical concern would be the concomitant solar elastosis which means damaged skin and excess sun exposure. This itself may be a conditioning for an MIS. We show a typical MIS below:



MIS is generally characterized by the following:

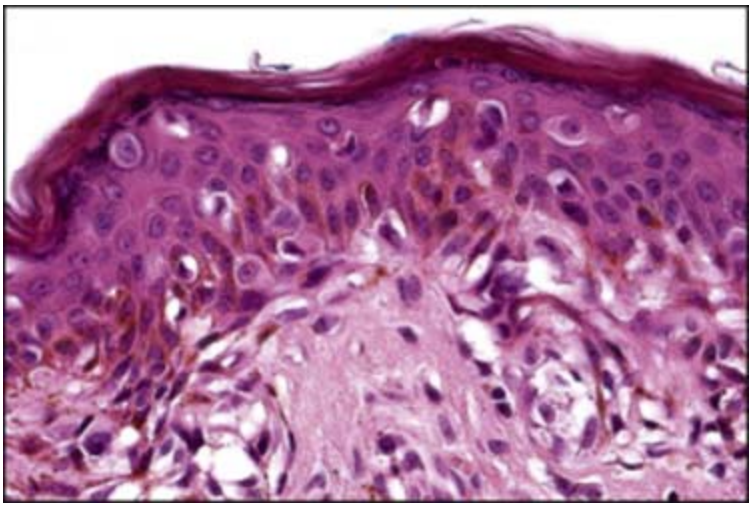
- irregular spread of tumor throughout the epidermis and or adnexal epithelium
- replacement of lower epidermis by tumor cells
- tumor cells in the most common presentation are usually large but variable in size, shape and irregularly displaced chromatin patterns

- nuclei are usually large and round but variable in size and shape with irregularly dispersed chromatin patterns
- large pink nuclei
- large nuclear/cytoplasmic ratios
- mitoses frequent and occasionally atypical
- dermal invasion absent

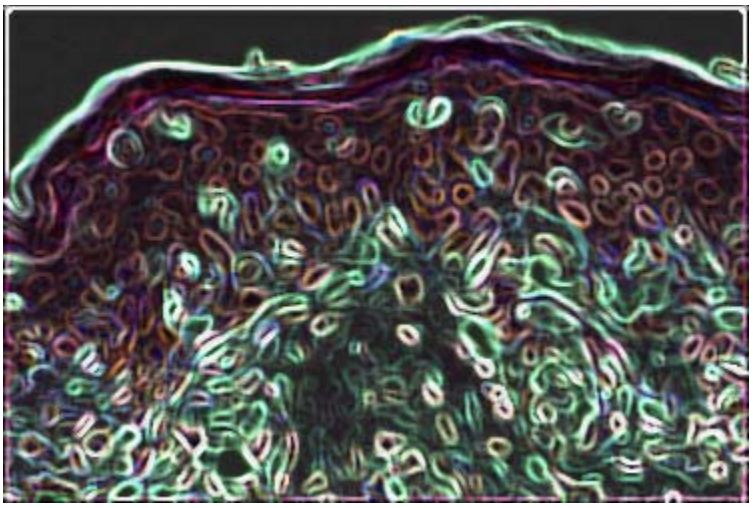
In addition we find the following in MIS:

- On microscopy, melanoma in-situ is confined within the epidermis. It may have melanocytes moving but restricted to the epidermis, thus little to any chance of metastasis.
- Features worrisome for melanoma in-situ include a predominance of single melanocytes, many single melanocytes higher up in the epidermis (instead of just in the basal epidermis, where they normally reside), and confluent, broad, irregularly sized and distributed nests of melanocytes.
- Often, the epidermal component is poorly demarcated (trailing off as single cells).
- Epidermal melanocytic proliferations that occur in the setting of significant solar elastosis should be reviewed with particular caution. The presence of MIS in sun damaged skin is a concern.

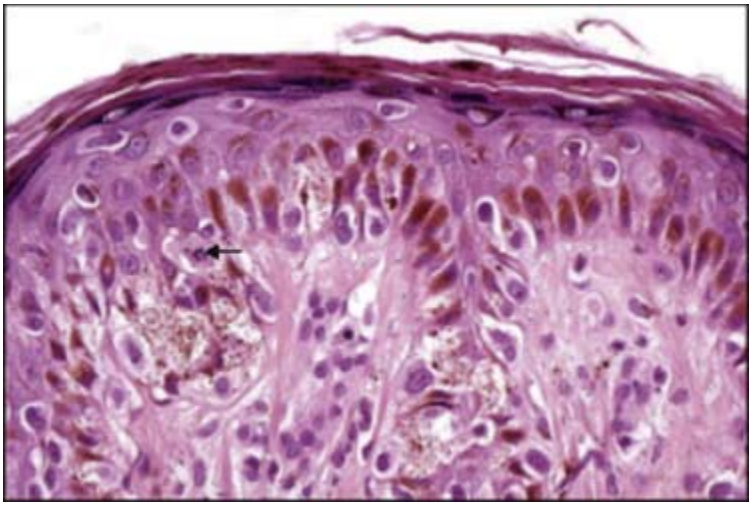
From McKee et al Fig 5.44 we have both MIS and a dysplastic nevus which we show below.



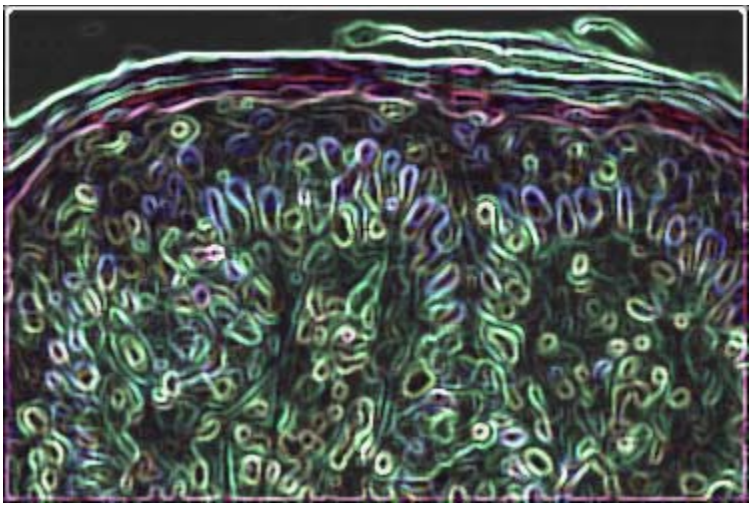
We have enhanced the color of the above in a way to attempt to further identify the melanocytes. This is shown below:



McKee et al in Fig 7.21 depict a MIS with a mitotic figure.

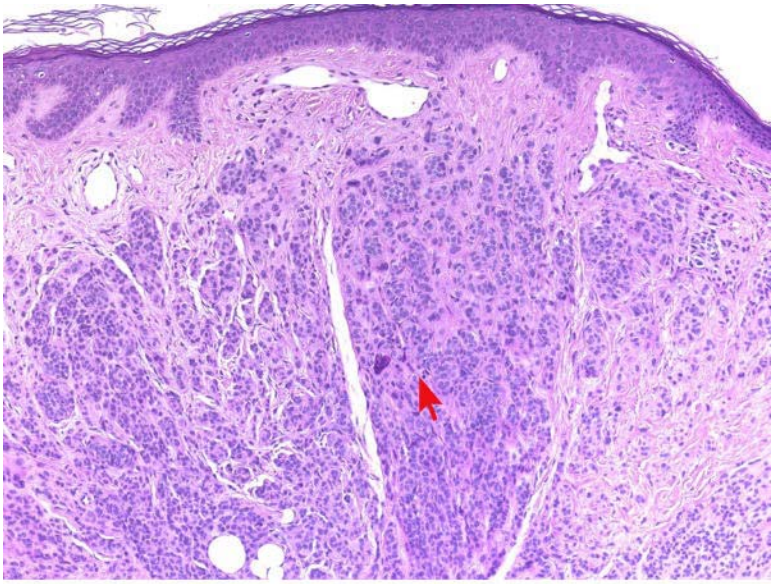


Again we enhance the color to further clarify the melanocytic expression.



Melanocyte histology is a challenging task. Oftentimes it is akin to finding a needle in a haystack. For example, there generally are few melanocytes at the basal layer of the skin. However when a cells takes on a malignant set of characteristics the melanocytes start to move. However, the location is not totally pathognomonic. We will look at this in the context of a standard set of melanocytes. For example the standard "mole" is based upon a set of clustered melanocytes, albeit of a well behaved manner. Whereas a melanoma in situ may be reflected by a few single wandering melanocytes in the epidermal layer away from the basal layer.

3.2.6 Intradermal Nevus



intradermal = only within the dermis

3.2.7 Basal Cell

Basal cell carcinoma is the most common form of skin cancer. As Rubin et al state[25]:

Basal-cell carcinoma characteristically arises in body areas exposed to the sun and is most common on the head and neck (80 percent of cases), followed by the trunk (15 percent of cases) and arms and legs. Basal-cell carcinomas have also been reported in unusual sites, including the axillae, breasts, perianal area, genitalia, palms, and soles. Nodular basal-cell carcinoma is the classic form, which most often presents as a pearly papule or nodule with overlying telangiectases and a rolled border, at times exhibiting central crusting or ulceration. Occasionally, nodular basal-cell carcinoma may resemble enlarged pores or pits on the sebaceous skin of the central portion of the face. Superficial basal-cell carcinoma presents as a scaly erythematous patch or plaque. Both nodular and superficial forms may contain melanin, imparting a brown, blue, or black color to these lesions.

The morpheaform type, also known as sclerosing, fibrosing, or infiltrative basal-cell carcinoma, typically appears as an indurated, whitish, scar-like plaque with indistinct margins. Suspicious lesions occurring in high-risk areas, such as the central portion of the face, should undergo prompt biopsy to obtain a timely diagnosis and to expedite definitive treatment. Skin biopsy will also identify amelanotic (nonpigmented) or minimally pigmented melanomas, which can sometimes mimic basal-cell carcinoma.

Rubin et al also state:

Inappropriate activation of the hedgehog (HH) signaling pathway is found in sporadic and familial cases of basal-cell carcinoma, medulloblastoma, rhabdomyosarcoma, and other tumors

We shall find the interaction with HH tobe of great significance as well proceed..

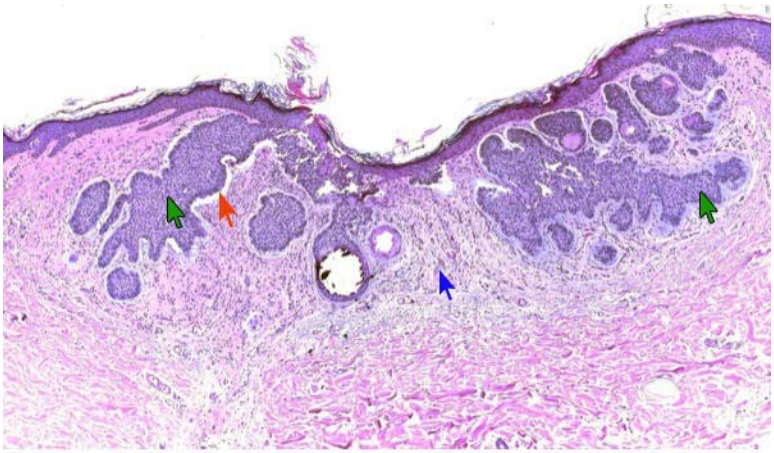
As Cowden et al state, the history of BCC and its timing in life are also of significance. Consider the following:

Basal cell carcinoma (BCC), first described by Jacob in 1827, is the most common malignant neoplasm of humans. After a rise in incidence of roughly 20% between 1971 and 1977, by 1998, roughly one million new cases were being diagnosed annually in the United States. In consequence of its high incidence, BCC, although eminently curable when the diagnosis is made promptly and the lesion treated in its early phase, constitutes an enormous financial burden on the healthcare system. Lesions occur on both sun-protected and sun-exposed skin but often have a different biology and morphology in these locations.

Typically, BCCs occur in the fourth decade of life and beyond, although exceptions to this occur, in particular, in the setting of specific genodermatoses or in patients with immune compromise.

[25] Rubin, A., et al, Basal Cell Carcinoma, NEJM, 353;21, November 24, 2005

As sun exposure plays a role in the development and transformation of BCC, patients with light skin phenotypes are particularly predisposed as expected; this includes in the context of blue eyes, red hair, and easy freckling as well as those whose occupational or leisure activities lead them to pronounced and prolonged sun exposure.

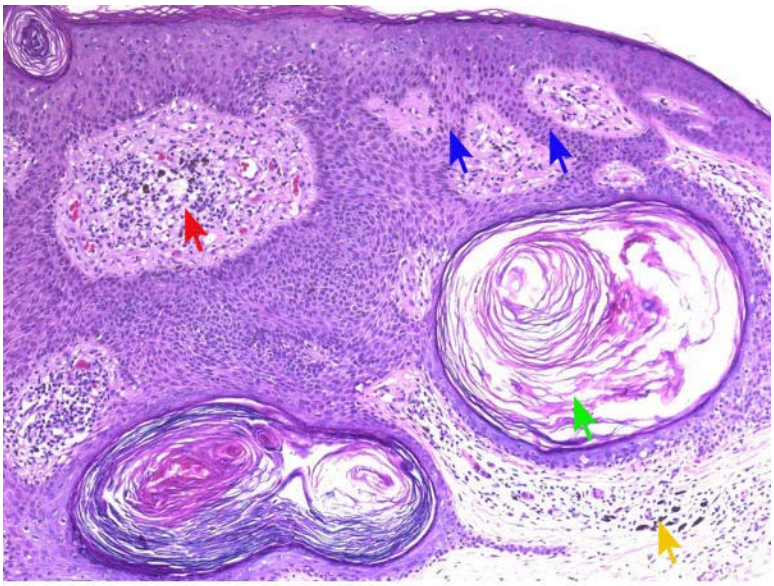


Although BCC is common, we shall not be focusing on it in any manner but the paper by Rubin et al is useful so as to understand the pathway issues which have some parallels.

3.2.8 Seborrheric Keratosis

Seborrheric Keratosis is a common benign skin lesion associated with aging. It has what is called a "stuck on" appearance and has a sebbacious feel. It has a well circumscribed margin and may be highly pigmented.

The figure below depicts a histological slide of a SB.



The SB can be characterized as follows:

- Microscopically there is an expansile plate-like growth of the epidermis with expanded and anastomosing rete ridges (the downward projections of epidermis that interdigitate with the dermis).
- There may be cysts that contain keratin and the lesions can show increased melanin pigmentation of the lesional keratinocytes.
- The histologic appearance is non-invasive with benign appearing cytologic characteristics.

3.2.9 Squamous Cell Carcinoma

Squamous Cell Carcinoma, SCC, is the second most common skin malignancy. We examine it briefly so that we can understand its basic principles. Unlike BCC, SCC, may have a more aggressive metastatic potential and may require extensive wide area excision. As Alan and Ratner state[26]:

Squamous-cell carcinoma is more likely to develop in injured or chronically diseased skin, including skin affected by long-standing ulcers, sinus tracts, osteomyelitis, radiation dermatitis,

[26] Alan, M., D. Ratner, CUTANEOUS SQUAMOUS CELL CARCINOMA, NEJM, Vol. 344, No. 13 March 29, 2001

or vaccination scars. Tumors arising at these sites may not be identified for years and, if neglected, carry a substantial risk of metastasis. Certain chronic inflammatory disorders may also predispose patients to the development of tumors; these disorders include discoid lupus erythematosus, lichen sclerosis, lichen planus, dystrophic epidermolysis bullosa, and lupus vulgaris (cutaneous tuberculosis).

They continue:

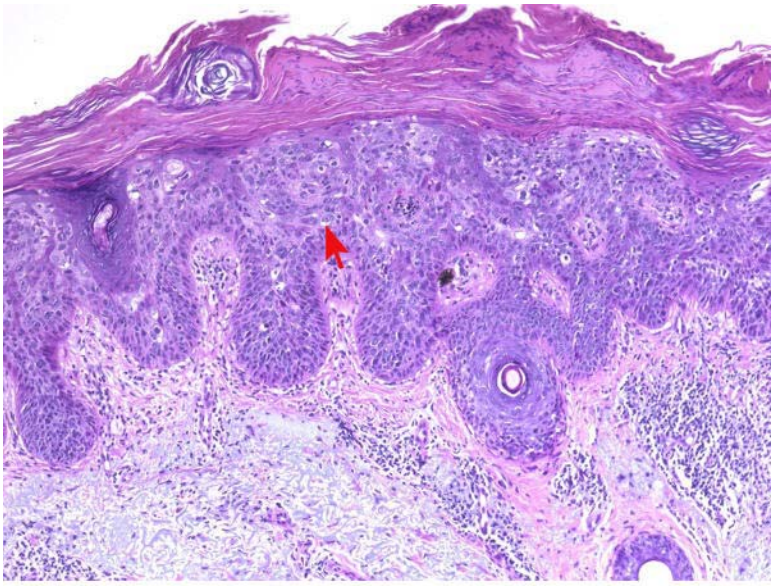
Squamous-cell carcinoma in situ may progress to invasive disease if not treated completely. The most common forms of squamous-cell carcinoma in situ are Bowen's disease and erythroplasia of Queyrat. Patients with Bowen's disease present with sharply demarcated, erythematous, velvety, or scaly plaques on sun-exposed areas (Fig. 6). Erythroplasia of Queyrat is less common and occurs on the glans penis of uncircumcised men as red, smooth plaques. Most invasive squamous-cell carcinomas occur on the head and neck; the next most common site is the trunk. The lesions are papules or plaques that are firm, skin-colored or pink, and smooth or hyperkeratotic. Ulceration may be present. [35] *Patients may describe their lesions as itchy or painful nonhealing wounds that bleed when traumatized.*

As Cowden et al state:

This type of squamous cell carcinoma occurs most commonly in sun- exposed areas of the body where there is usually clinical evidence of sun damage, or "dermatoheliosis". The precursor lesion, the actinic keratosis, presents mainly on the face, arms, and dorsum of the hands, on the superior aspect of the ear, and on the scalp, particularly in bald men. These lesions are usually slightly erythematous, are sometimes flesh-colored, and usually have a scale that is whitish and densely adherent to the skin. Some are markedly hyperkeratotic and resemble seborrheric keratoses or verruca vulgaris. There is a type that is rather fl at and variably pigmented that gradually expands, termed the superficial pigmented actinic keratosis (SPAK).

A squamous cell carcinoma that arises in actinic keratosis is first identifiable as an area of thickening of the precursor lesion. This thicken-ing with some increase in erythema can lead to a hyperkeratotic lesion that has a densely adherent crust initially. As the lesion progresses the thickened area may give rise to a nodule or may become ulcerated, manifesting a central crust.

These lesions are often associated with a history of bleeding after trauma, especially after washing and abrading the area with a wash cloth or towel. In the presence of a SPAK, the lesion may be pigmented and may resemble a melanoma.



Intraepidermal atypical squamous cells

+

We can characterize the above as follows:

- Microscopically squamous cell carcinoma in situ shows replacement of normal maturing epidermal keratinocytes with intraepidermal atypical squamous cells that do not show maturation and appear disordered.
- Invasive squamous cell carcinoma shows irregular sheets or islands of atypical squamous cells that originate from the overlying epidermis and invade the dermis directly.
- Sometimes vascular invasion or perineural invasion can be seen.

3.3 MELANOMA

Melanoma is simply the uncontrolled growth, spread, and creation of immortal distorted melanocytes which have the capability to spread throughout the body and destroy the functioning of other normal benign cells. Malignant metastatic melanoma may spread to the lungs, brain, liver or other parts of the body, destroying the normal cells and in turn destroying the functions of the normal organ. In the brain the metastatic cells create clusters which push out the normal cells.

In a 1930 NEJM paper McKittrick states[27]:

Melanotic sarcoma. Melanotic sarcoma, or melanoma, may be quite varied in its clinical manifestations. It frequently arises in pigmented moles. Rarely is it noted in the intestinal tract. It may arise in any part of the body where there is pigmentation, and even in parts of the body where one does not commonly expect pigmentation.

[27] McKittrick, L., Malignant Disease of the Skin, NEJM, Vol 202 No 15, pp 725-726, April 10, 1930.

Metastases as a rule are first to skin, later to the regional lymph nodes, lymph nodes elsewhere in the body, and ultimately, to nearly every organ in the body. Bony metastases are very rare. It is very difficult to determine which mole is sometime going to become a melanotic sarcoma and which one is not. Any pigmented lesion which shows any change in any way should be removed. Practically all of these lesions will show some change in character or in size before going into the malignant, metastasizing lesion, and if removed then, the patient may be cured. Another factor in deciding whether to remove or not is the location.

If the person has a pigmented mole or lesion which is located where constant irritation is unavoidable, I believe it should be removed unless it is so large that removal would be too disfiguring. There are two things to do for these pigmented moles on the skin. One is to leave them alone until there is some definite change or until something about them makes you feel

And the other is to take them suspicious out and to take them out well. Use no radium. No superficial cauterization. No diathermy. Take them out well with a wide margin of normal skin as though the lesion were already malignant. Leave them alone or else treat them right.

This recommendation is prescient. Namely, the physician should perform a wide area excision of any suspicious mole. There have been many cases, from then, even to the present, where the removal failed to be done properly. That of course has led to excess mortality.

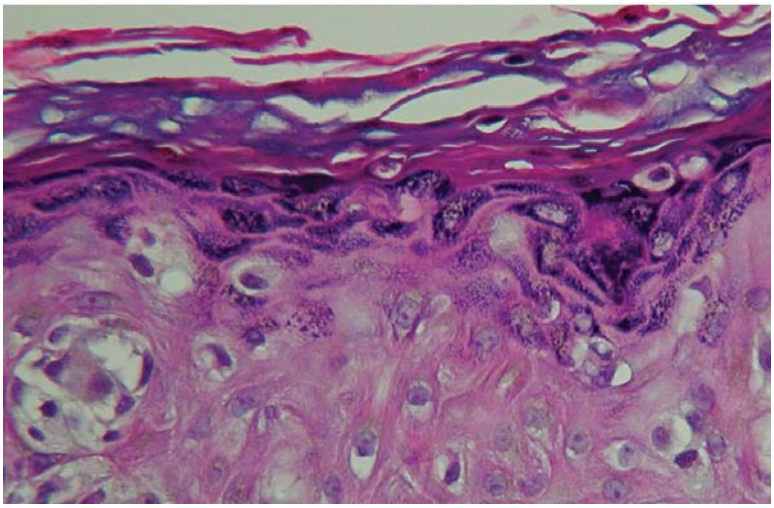
As Crowson et al state:

Superficial spreading melanoma constitutes approximately 70% to 75% of all melanomas, and, though mainly found on the trunk and the sun-exposed areas of the upper arms and the lower legs, it may occur anywhere on the body surface... Therapeutic ultraviolet (UV) irradiation and use of tanning bed may play an etiological role. A Norwegian melanoma project carried out between 1989 and 1993 revealed that persons with red hair and skin type I/II were at increased risk of develop-ing melanoma. Intermittent intense exposure to UV irradiation as in the context of beach vacation in adolescence, prior sun burns, or exposure to sunbeds and sunlamps is associated with increased risk, while chronic sun exposure may be protective.

Clinically, the incipient lesion is a dark-brown macule, a few milli-meters in size that gradually enlarges to reach an average size of 2.5 cm. However, marked variation in size may be noted with some lesions being as small as 4 mm and others as large as 8 to 10 cm. When fully evolved, the lesion has variegated color and irregular borders. The onset of the vertical growth phase is associated with the presence of a distinctive nodule.

Superficial spreading melanoma arises de novo in approximately 75% of the cases and, in the remainder, it is associated with a pre-existing nevus, including those of congenital and dysplastic nevus subtypes. Melanoma arising in a pre-existing dysplastic nevus is usually of superficial spread-ing type.

Cowden et al show the SSM radial phase as follows:

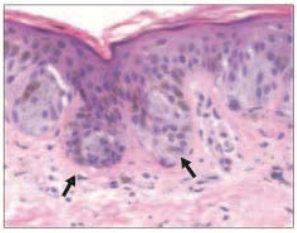
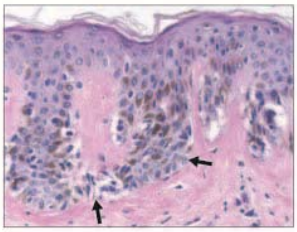
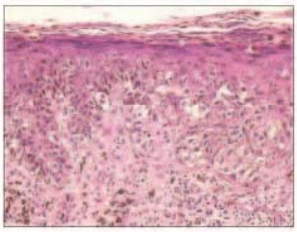
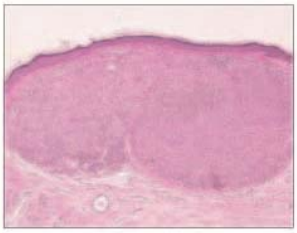
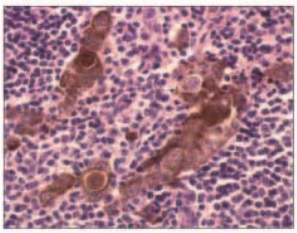


Regarding the above Cowden et al state:

Cells with an epithelioid cytomorphology grow in a haphazard, pagetoid fashion with single-cell dispersal through the epidermis to the cornified layer. The cells exhibit prominent nucleolation and high nuclear to cytoplasmic ratios. There is significant variability with respect to nuclear size and shape.

Note that pagetoid means upward spreading. One can see the large nucleoli clear melanocytes spreading upward to the top epidermal layer and we can see the irregular clusters and the irregular size as well as large nucleoli to cytoplasm ratios in all of these cells. This shows many of the general characteristics of all cancers; growth (increasing cell size), uncontrolled proliferation (mitotic activity), loss of location, namely the cells are not where they are supposed to be.

From Miller and Mihm we have the following Table which present examples of each type:

Histopathological Appearance	Description	Histologic Features
 Benign nevus	**Step 1** The first event is a proliferation of structurally normal melanocytes leading to the benign nevus. Clinically, these nevi present as flat or slightly raised lesions with either uniform coloration or a regular pattern of dot-like pigment in a tan or dark brown background. Histologically, such lesions have an increased number of nested melanocytes along the basal layer (arrows).	Proliferation of melanocytes Benign lesions
 Dysplastic nevi	**Step 2** The next step is the development of aberrant growth. This may occur within a preexisting benign nevus or in a new location. Clinically such lesions may be asymmetric, have irregular borders, contain multiple colors, or have increasing diameters. Histologically, such lesions have random and discontiguous cytologic atypia (arrows).	Dysplastic cells Random atypia
 Radial-growth phase	**Step 3** During the radial-growth phase, cells acquire the ability to proliferate intraepidermally. Clinically, they sometimes present as raised lesions. These lesions no longer display random atypia and instead show cytomorphologic cancer throughout the lesion. In addition to the intraepidermal cancer, the cells can penetrate the papillary dermis singly or in small nests but fail to form colonies in soft agar.	Intraepidermal growth Continuous atypia
 Vertical-growth phase	**Step 4** Lesions that progress to the vertical-growth phase acquire the ability to invade the dermis and form an expansile nodule, widening the papillary dermis. The cells can also extend into the reticular dermis and fat, are capable of growth in soft agar, and have the capacity to form tumor nodules when implanted in nude mice.	Dermal invasion
 Metastatic melanoma	**Step 5** The final step in the model is the successful spread of cells to other areas of the skin and other organs, where they can successfully proliferate and establish a metastatic focus. These cells can grow in soft agar and can form tumor nodules that may metastasize when implanted in nude mice.	Metastasis

3.3.1 Example of Metastasis

Malignant Melanoma is a highly invasive cancer and has the propensity to metastasize to the brain as the primary site. This paper looks at the literature and evaluates the propensity of such metastases to result in psychotic behavior as the first presenting symptom. This paper focuses

especially on those patients presenting after multiple recurrences of melanoma with extensive resection and with a recurrence on the head or neck. There is a significant body of literature linking the incidence of brain metastasis and extensive head and neck melanoma and there is also another body of literature linking brain tumors, including metastasis, to psychiatric changes.

We now look at several issues regarding malignant melanoma. These issues relate to the question of how frequently melanoma metastasizes to the brain and under what conditions and then in those metastasized lesions what are the presenting symptoms and finally how frequently the metastatic lesions may result in psychotic behavior. We approach this review by looking at each of the issues in some detail and then we proceed to review the literature to substantiate what is currently known in the field.

Table 1 AJCC 2002 Melanoma Staging

AJCC 2002 Revised Melanoma Staging				
		Overall Survival		
		1-year	5-year	10-year
0	Intraepithelial/in situ melanoma (TisN0M0)		100%	100%
IA	≤ 1 mm without ulceration and Clark Level II/III (T1aN0M0)		95%	88%
IB	≤ 1 mm with ulceration or level IV/V (T1bN0M0)		91%	83%
	1.01-2 mm without ulceration (T2aN0M0)		89%	79%
IIA	1.01-2 mm with ulceration (T2bN0M0)		77%	64%
	2.01-4 mm without ulceration (T3aN0M0)		79%	64%
IIB	2.01-4 mm with ulceration (T3bN0M0)		63%	51%
	> 4 mm without ulceration (T4aN0M0)		67%	54%
IIC	> 4 mm with ulceration (T4bN0M0)		45%	32%
IIIA	Single regional nodal micrometastasis, nonulcerated primary (T1-4aN1aM0)		69%	63%
	2-3 microscopic regional nodes, nonulcerated primary (T1-4aN2aM0)		63%	57%
IIIB	Single regional nodal micrometastasis, ulcerated primary (T1-4bN1aM0)		53%	38%
	2-3 microscopic regional nodes, ulcerated primary (T1-4bN2aM0)		50%	36%
	Single regional nodal macrometastasis, nonulcerated primary (T1-4aN1bM0)		59%	48%
	2-3 macroscopic regional nodes, nonulcerated primary (T1-4aN2bM0)		46%	39%
	In-transit met(s)/satellite lesion(s) *without* metastatic lymph nodes (T1-4a/bN2cM0)		30-50%	
IIIC	Single microscopic regional node, ulcerated primary (T1-4bN1bM0)		29%	24%
	2-3 macroscopic regional nodes, ulcerated primary (T1-4bN2bM0)		24%	15%
	4 or more metastatic nodes, matted nodes/gross extracapsular extension, or in-transit met(s)/satellite(s) *and* metastatic nodes (any TN3M0)		27%	18%
IV	Distant skin, subcutaneous, or nodal mets with normal LDH (any T any NM1a)	59%	19%	16%
	Lung mets with normal LDH (any T any NM1b)	57%	7%	3%
	All other visceral mets with normal LDH or any distant mets with increased LDH (any T any NM1c)	41%	9%	6%

A great deal of research has been done on the genetic fabric of melanoma. There is no simple answer as to the genetic initiation but the recent work by Curtin et al details the many genetic underpinnings of the malignancy. Specifically Curtin et al concluded in their recent paper:

"Knowledge of the genetic differences among melanomas could be valuable in the design of therapeutic strategies. Our results lead us to make a prediction. The group of tumors on skin without chronic sun-induced damage, which represent the most common type of melanoma, frequently had a mutation in BRAF together with a loss of PTEN or mutations in N-RAS alone. Thus, they would be expected to be responsive to therapeutic interventions targeting the RAS–RAF–ERK and PI3K pathways. In contrast, the majority of melanomas in the other three groups did not have mutations in BRAF or N-RAS but instead had increased numbers of copies of the downstream gene CCND1 or CDK4.

Thus, these three groups of melanomas would be less likely to respond to therapeutic interventions that target upstream components of the mitogen-activated protein kinase pathway including BRAF, such as sorafenib. Our study provides genetic support for the existence of distinct molecular pathways to melanoma, each with a unique relationship to exposure to ultraviolet light. This finding should affect the design of future studies involving the treatment and prevention of melanoma and suggests the existence of as yet- unidentified susceptibility factors."

The results of Curtin seem to imply that the sun damaged generation of melanoma may be more difficult to treat than the non-sun damaged variety, specifically due to the origin from differing genetic pathways. Second, the approach taken by Curtin et al may raise the question as to the MPM syndrome.

As to the MPM and genetic markers relationship, Ferrone has stated:

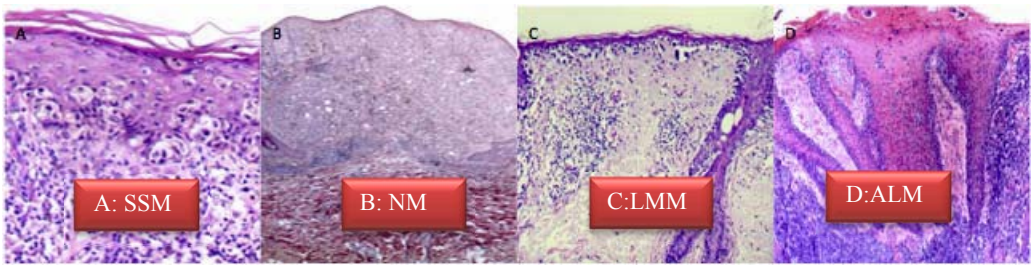
"Several risk factors associated with the development of MPM have been identified. These include a positive family history of MPM and a personal history of dysplastic nevi (DN); atypical moles that are risk markers but non-obligate precursors of melanoma. Among patients with MPM, 18% to 38% are reported to have a positive family history of melanoma and 38% to 46% are reported to have a history of dysplastic nevi. However, few longitudinal cohort-defined databases have prospectively recorded known risk factors for all patients with melanoma to assess the impact of these risk factors on the development of MPM."

3.3.2 Types of Melanoma

We consider the classic histological forms of melanoma. The primary indicator is the movement of the melanocytes from the basal layer and the change in the gross appearance of the melanocyte.

The following is from Eichhoff[28]:

[28] http://e-collection.library.ethz.ch/view/eth:1923?q=melanoma



The authors characterize the above as follows:

Immunohistopathological characteristics of melanoma subtypes.

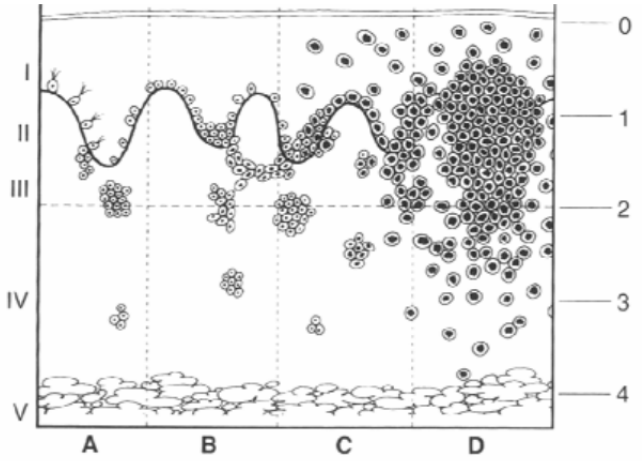
(A) SSM is an epidermal spreading tumor with atypical melanocytes displaying pagetoid growth in the upper epidermis.

(B) NM is a pigmented lesion composed of nests of proliferating cells in both epidermis and dermis.

(C) LMM is a confluent growing lesion (Lentigo maligna) with evidence of epidermal appendages and dermal growth.

(D) ALM presents lentiginous and nesting proliferation of atypical melanocytes often with some evidence of pagetoid growth. This example is ulcerated with a thickened papillary epidermis and has signs of acanthosis.

The level of the invasion of the malignant cells is given by a Clark or Breslow level. These are now standard measure and they reflect the depth of penetration of the lesion. They are used because they correlate well with a prognosis. We demonstrate an example below where we have disease states from A through B and Clark and Breslow levels.



The left hand above measures the Clark level and the right hand side the Breslow level. We use these for an assessment of primary melanoma lesion growth[29]. Clark's level (left) includes epidermal (I), dermal (II-IV) and subcutaneous tissue (V) compartments.

Breslow thickness (right) is measured in millimeters from the top of the lesion to the deepest point of invasion.

As Miller and Mihm state[30]:

The Clark model of the progression of melanoma emphasizes the stepwise transformation of melanocytes to melanoma. The model depicts the proliferation of melanocytes in the process of forming nevi and the subsequent development of dysplasia, hyperplasia, invasion, and metastasis. Numerous molecular events, many of them revealed by genomic and proteomic methods, have been associated with the development of melanoma. But rather than catalogue all the molecular lesions in this tumor, we will focus on connections between molecular pathways and risk factors for melanoma, the different steps of neoplastic transformation, and the patterns of molecular changes in melanoma .

Specifically they reiterate the use of the now classic Clark model but they do emphasize the need for added and/or enhanced methods. Unfortunately they are generally not used nor are they available.

Let us consider the Clark model for formation. Again from Miller and Mihm we have:

In the Clark model, the first phenotypic change in melanocytes is the development of benign nevi, which are composed of neval melanocytes. The control of growth in these cells is disrupted, yet the growth of a nevus is limited — a nevus rarely progresses to cancer.

The absence of progression is probably due to oncogene-induced cell senescence, in which growth that is stimulated by the activation of oncogenic pathways is limited.

At a molecular level, abnormal activation of the mitogen-activated protein kinase (MAPK) signaling pathway (also called extracellular-related kinase [ERK]) stimulates growth in melanoma cells. Activation of this pathway is the result of somatic mutations of N-RAS, which are associated with about 15 percent of melanomas, or BRAF*, which are associated with about 50 percent of melanomas.*

These mutations, which occur exclusively of each other, cause constitutive activation of the serine–threonine kinases in the ERK–MAPK pathway. BRAF *mutations occur at a similar frequency in benign nevi and in primary and metastatic melanomas.*

Since most nevi cease proliferation and remain static for decades, these similar frequencies suggest that nevi must acquire additional molecular lesions to free themselves of growth restraints and become malignant. Experiments in model systems support this hypothesis. In

[29] Adapted from (Houghton & Polsky, 2002).

[30] Miller, A., M. Mihm, Melanoma, NEJM, 355;1 July 6, 2006

zebrafish, melanocyte-specific expression of a mutant BRAF protein causes an ectopic proliferation of melanocytes, analogous to human nevi.

In these fish, the combination of a BRAF *mutation and inactivation of the tumor-suppressor* gene p53 *causes melanocytes to become malignant.*[22] *In human melanocytes, mutant BRAF protein induces cell senescence by increasing the expression of the cell-cycle inhibitor of kinase 4A (INK4A). INK4A limits hyperplastic growth caused by a* BRAF *mutation. The arrest of the cell cycle caused by INK4A can, however, be overcome by mutations in INK4A itself, as well as other cell-cycle factors.*

Histological assessment cues include some of the following:

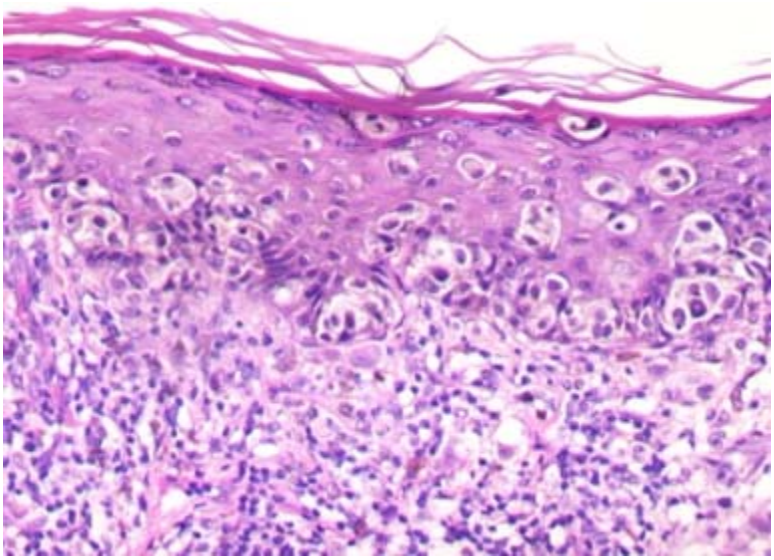
(A) normal melanocytes as isolated individuals at the epidermal-dermal junction or as small clusters comprising benign nevi,

(B) proliferation of atypical melanocytes indicating an atypical nevi, these generally show large nucleus type cells, small cytoplasm, and multiple melanocytes in clusters with dramatically differing shapes.

(C) Radial Growth Phase melanoma, which means a spreading out from a center or central area but lacking yet in any substantial depth

(D) Vertical Growth Phase melanoma. This phase shows substantial depth penetration. Generally if there is substantial, or even minimal, vertical growth we may highly suspect metastatic growth and spread.

We demonstrate a simple superficial spreading melanoma in generally radial phase below.



Superficial spreading melanoma (SSM) is the most frequent histological type of melanoma (about 65% of all cases). It is characterized by the presence of a radial growth phase mostly

composed of large epitheliod melanocytes haphazardly distributed at all levels of the epidermis in a pagetoid fashion.[31]

We will discuss the general histology of the skin including melanocytes and melanoma. For years histology has been the primary mode of diagnosing the disease and it has had its challenges since some of the pigmented lesions examined on a microscopic scale may or may not be readily identified as a melanoma. We shall try to present the cell presentation in its normal form and then attempt to establish a generic evolution as see microscopically as melanoma develops. The focal point is the melanocyte and its normal state.

The purpose of exploring histology is to understand shape and location, two of the characteristics modified when a melanocyte becomes malignant. The cell loses its morphology and it loses its ability to stay where it is supposed to stay. This will be an important factor in understanding the development and progression of a melanoma.

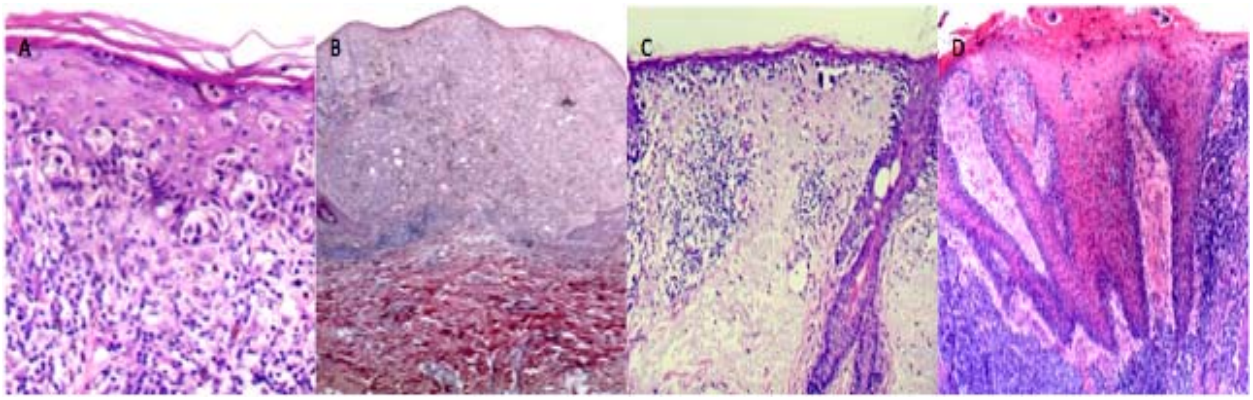
The following depicts several example of melanoma, not just superficial spreading. Specifically it demonstrates the immune-histo-pathological characteristics of melanoma subtypes. Specifically:

(A) Superficial Spreading Melanoma (SSM) is an epidermal spreading tumor with atypical melanocytes displaying pagetoid growth in the upper epidermis. The SSM is often in a spreading mode horizontally in the dermis and basal layer before any vertical growth stage starts.

(B) Nodular Melanoma (NM) is a pigmented lesion composed of nests of proliferating cells in both epidermis and dermis. This is the most aggressive form of melanoma and it has a very aggressive vertical stage.

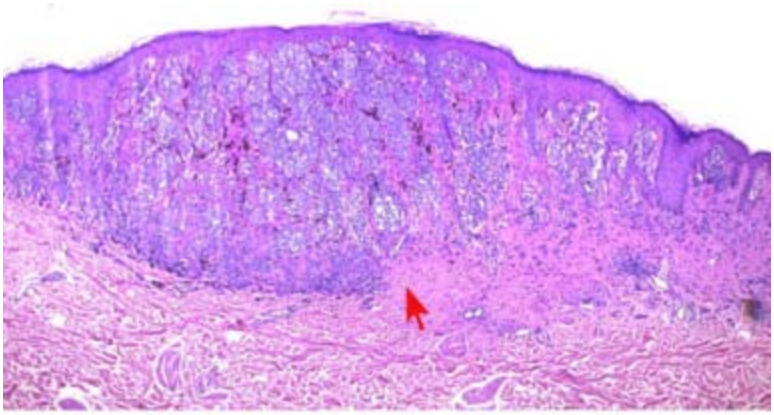
(C) Lentigineous Malignant Melanoma (LMM) is a confluent growing lesion (Lentigo maligna) with evidence of epidermal appendages and dermal growth.

(D) Acral Lentigous Melanoma (ALM) presents lentiginous and nesting proliferation of atypical melanocytes often with some evidence of pagetoid growth. This example is ulcerated with a thickened papillary epidermis and has signs of acanthosis.



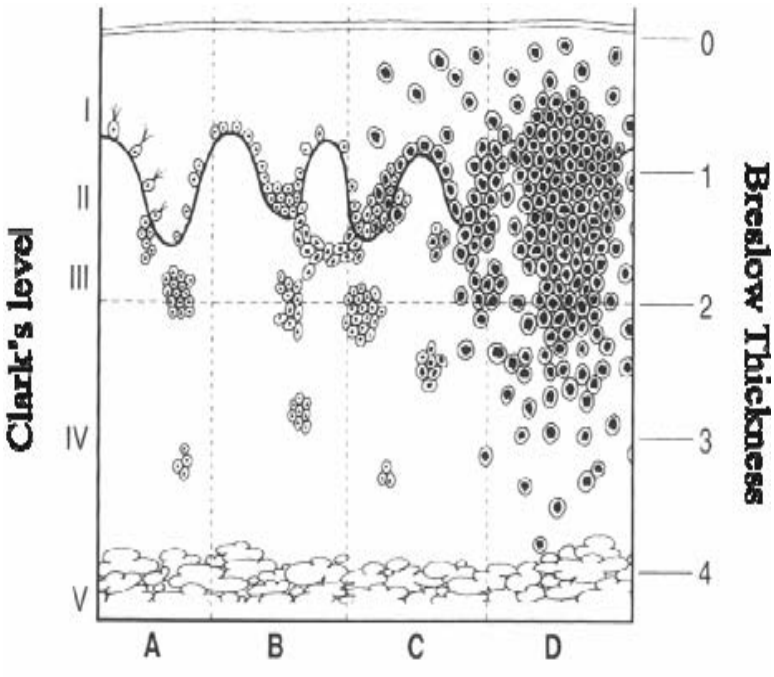
[31] http://www.mmmp.org/MMMP/import.mmmp?page=pathology.mmmp

An SSM is shown in some detail below:



Note asymmetry

Melanoma demonstrates a progression. In SSM melanoma we have the model shown below. We can see the a*ssessment of primary melanoma lesion growth*. Clark's level (left) includes epidermal (I), dermal (II-IV) and subcutaneous tissue (V) compartments. Breslow thickness (right) is measured in millimeters from the top of the lesion to the deepest point of invasion.

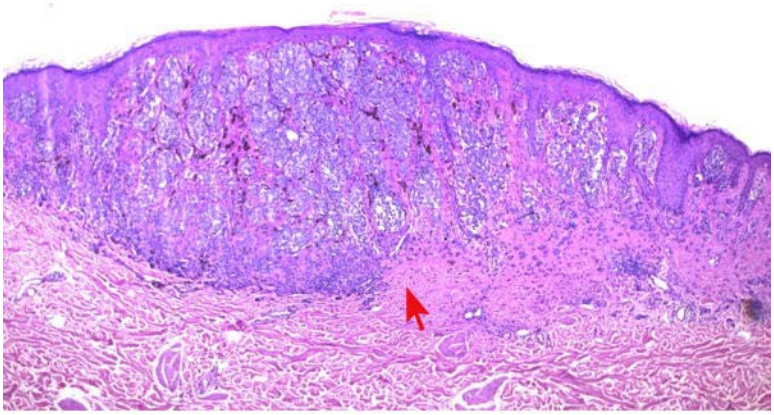


But note that we see the progression as movement first downwards, into the dermis, with clustered of melanocytes. This is distinct from MIS which goes upwards to the epidermis above the basal layer.

3.3.3 Melanoma Histology

We briefly will discuss some of the major histological characteristics. We consider a superficial spreading melanoma (SSM), the most common. The characteristic will later be correlated with the genetic control paths. It will be important to try to relate the histological characteristics with what pathways control them and in turn what pathway aberrations lead to the aberrations noted in the histological characteristics.

We can also look at the melanoma slide below and examine its characteristics:



Let us examine SSM. SSM is characterized by:

1) It is like melanoma in situ for epidermal segment

2) It exhibits invasion of papillary dermis by single cells or nests of cells with similar morphology to those found in epidermis

3) There are aggregates of cells in papillary dermis and extending more deeply composed of cells with often differing morphologies from the intraepidermal cells

4) The invasive cells are commonly round with large nuclei and prominent nucleoli

5) The nuclear chromatin is often coarse and irregularly disposed

6) The cytoplasm dusky pink and filled with fine melanin granules

7) There appears to be dermal mitoses often present and may be atypical.[32]

[32] Note from Gartner and Hiatt: "**Mitotic figures** should be common in the stratum basale because this layer is partially responsible for cell renewal in the epithelium. However, mitosis occurs mostly during the night, and histological specimens are procured during the day; thus, mitotic figures are rarely seen in histological slides of skin. When new cells are formed via mitosis, the previous layer of cells is pushed surfaceward to join the next layer of the epidermis, the stratum spinosum."

8) There is an inflammatory host response; common beneath intraepidermal component but variable beneath dermal tumor. One can see a clustering of T cells and other white cell components.

To the above we can add a significant set of standard histological features which have been used to characterize a melanoma. The following are the most common and may not all be present.

1) Asymmetry and poor circumscription: The loss of growth control exhibited by significant asymmetry is common.

2) Conspicuous junctional activity: The melanocytes at the junction between the epidermis and dermis show significant growth, often of a chaotic nature.

3) Junctional cytoplasmic and nuclear pleomorphism: Distortions in the nucleus, excessive size, as discussed below, shape and form.

4) Prominent nucleoli: Large and prominent nucleoli are frequent characteristics. One can see the clear melanocyte cytoplasm but the nucleoli is the dominant observation.

5) High nuclear-cytoplasmic ratio: A consequence of what we have discussed above.

6) Junctional mitotic activity: Clear expression of mitosis at the junction. This means that the cell is replicating and that it may very well be doing so in an erratic and uncontrolled manner. We shall discuss this in detail later.

7) Dermal mitotic figures: Mitotic numbers may very well exceed normal levels.

8) Abnormal mitoses: Mitotic change is common. It will beg the issue of a melanoma stem cell but this we shall discuss at length later.

9) Ascent of tumor cells into the upper layers of the epidermis (pagetoid spread)

10) Absence of maturation of the dermal component

11) Lower border often has a pushing growth pattern

12) Dermal nodule formation: The nodule formation may exhibit the pushing downward and upward and appear superficially also as nodular.

13) Spindled or epithelioid cells in dermal component

14) Variable melanin pigment: Melanasome production can be variable as cells may no longer function is a normal fashion. Thus pigmentation may vary abruptly and one may eve see what appears to be a regression.

As we proceed, we will from time to time refer to these histological characteristics. However, our goal will be to try to describe how these histological characteristics are reflective of pathway abnormalities. Ideally one would like to use histological analysis as a secondary approach, using a genetic profile for diagnostic and prognostic tools.

3.4 CONCLUSIONS

We have presented an overview of the histological structure of benign and malignant melanocytes. This represents what we can now call a classic approach to diagnosis, by examining the gross structure of melanocytes, their proliferation, growth and location. For decades this has been the primary and almost sole manner of diagnosing melanoma. From a prognostic perspective this also has been used with the Breslow and Clark measures as a way to stage melanoma and to ascertain treatment options, limited as they may be.

Specifically the current state of diagnosis is best phrased by Gershenwald, M.D., and Merrick I. Ross[33]:

Biopsy of a suspicious pigmented lesion followed by histologic assessment provides definitive diagnosis and the essential primary tumor information (i.e., tumor thickness, ulceration, and mitotic rate). Since this information defines the disease stage and predicts the risk of occult regional lymph-node metastases, it influences treatment recommendations.

However, we also now know that having a genetic profile of the melanoma helps considerably. The process of obtaining that profile is complex and open to some controversy as well shall discuss[34]. The use of classic histological methods are still the most well accepted.

The paper by Smalley and Sondak does look at these genetic markers as added insight. They state:

Nonetheless, the data provided by Flaherty and colleagues represent a major advance in the treatment of metastatic melanoma. But what's next? How much can we improve on these results — especially in terms of extending the duration of dis ease control — through combination therapy or even by manipulating the dose and schedule of single-agent therapy?[13] When should this therapy be moved to the adjuvant setting? How can we achieve similar success in treating patients with wild-type BRAF? The prospects for patients with metastatic melanoma have never been brighter, but the need for further progress through laboratory research and well-conducted clinical trials is as great as — or greater than — ever.

Thus there is a great deal of confidence that we can seek out and use highly effective genetic markers to enhance or even replace histological examinations.

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[33] Gershenwald, J., M. Ross, Sentinel-Lymph-Node Biopsy for Cutaneous Melanoma, NEJM, 364;18, may 5, 2011.

[34] Smalley, K., V. Sondak, Melanoma — An Unlikely Poster Child for Personalized Cancer Therapy, NEJM, 363;9 nejm.org August 26, 2010

4 THE CELL CYCLE: A BEGINNING FOR PATHWAYS

Cancer is basically uncontrolled cell growth, replication, and failure for cells to die off, normal apoptosis. It may also include loss of location stability and metabolic enhancement, but let us start with the key issue, replication. Then we examine two other major factors; apoptosis or cell death and cell to cell adhesion, or simply cells being where they should be.

Cancer in many ways is a loss of the three factors:

1. Cell Replication: This is the normal or abnormal cell cycle.

2. Cell Death: This is normal cell death or apoptosis.

3. Cell Localization: The establishment and maintenance of a cells relative position and function.

We shall thus begin with the control of the cell cycle and then work upwards in terms of the cells control mechanism.

The following Figure presents a simple view of how cell signalling functions. There are six functions described, and not all must be present in any cell function. The steps are generally:

1. Ligand: There is some external activator that floats about and ultimately finds its home on the surface of a cell. Now the issue is not that there is one such protein floating about that eventually may find itself attached to the surface of a cell. The protein may be from afar or it may be from the very same cell. We could then consider the concentration of the protein as well, and its flow across cells themselves as well. This issue is a complex one and all too often it is treated like a simple one protein to one receptor issue. In reality it is a distributed random process.

2. Receptor: The ligand seeks and may ultimately find a receptor. The receptor is a protein on the cell surface. A cell produces the protein and the number of such receptors may be significant as well. Thus there exists a concentration in space of the ligands and they can attach to and activate receptors, proteins, on cell surfaces.

3. Adaptor: The Receptor when connected to a ligand effects a response and there may be an adaptor protein which then gets connected and starts the inter-cell communications process.

4. Transducer: The transducer, such as RAS or PI3K, converts the signal to the receptor as displayed by the adaptor into the beginning of a chain down through the cytoplasm. This is a highly controlled and redundant chain which can become unstable if certai genes are affected and the controlling proteins disabled.

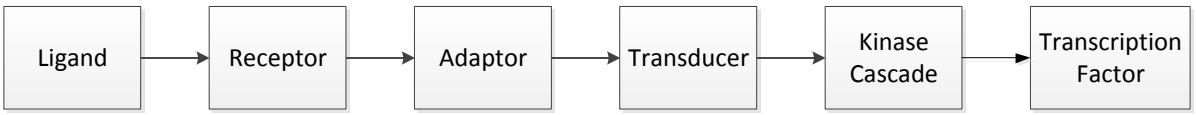
5. Kinase Cascade: This is the chain of protein communicating links and effectors from the Transducer to the cell nucleus and includes the initiation of the targeted transcription factor. As with the Transduce this kinase chain is controlled by redundant checks but if they become defective then the chain internal controls can be lost and the result become unstable.

6. Transcription Factor: This is the protein which has been activated within the nucleus which then commences transcription of the targeted sets of genes for the purpose of producing the resulting product.

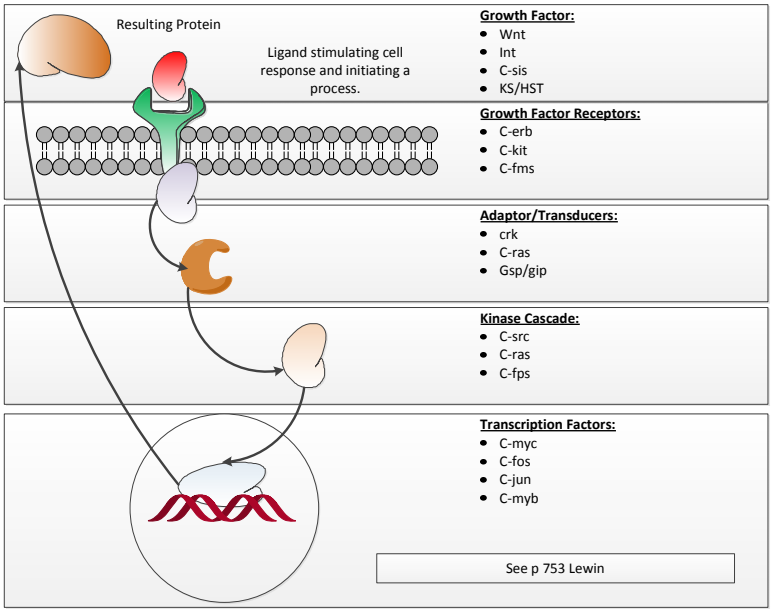
Note that this is a complex process.

Ligand	PDGF	Insulin	Growth Hormone	IL-1β	TGF-β
Receptor	PDGF Receptor	Insulin Receptor	GH Receptor	IL Receptor	TGF Receptor
Adaptor	SHP2/Grb2	IRS 1			
Transducer	SOS/Ras	PI3K	JAK	JAK	Type 1 Receptor
Kinase Cascade	MAPK	Akt			
Transcription Factor	Ternanry complex factors	FOXO	STATs	STATs	SMADs

See p 818 Lewin



The following depicts the process at several levels in a cell.



Now there are two major states a cell finds itself in; stasis and reproduction. A third, apoptosis, is natural cell death, we shall consider later. In stasis the cell is in G0 and producing proteins generally in response to external ligands or through normal internal processes. Unlike most

standard biological models, we look at the proteins generally in terms of their concentrations and thus look at cell kinetics as well.

A cell in stasis is a little protein production factory, and each cell is pumping out the proteins and they then are in some extracellular balance. The cells in stasis communicate with one another via their respective ligands. In contrast when a cell reproduces it is standing out from the crowd if one will and looking out for itself.

We now examine first gene operations and then cell replication.

4.1 GENETIC PRINCIPLES AND APPLICATIONS

This section is a brief review of some of the basic principles of genetics necessary for understanding cancer development and progression.

The genetic operation of a cell and its impact on the homeostatic functioning requires an understanding of a few essential facts from the now well understood operations of the gene and the secondary pathways associated with them. This Chapter is a review of these principles. Specifically we review the following:

1. Gene structure and operation. This includes the basic Watson and Crick model as is currently understood. The development that we use is a functional model and note one that would be more familiar to the biologist. In all our analyses we will build models of functions and leave the basic principles and their modifications to the bench scientist.

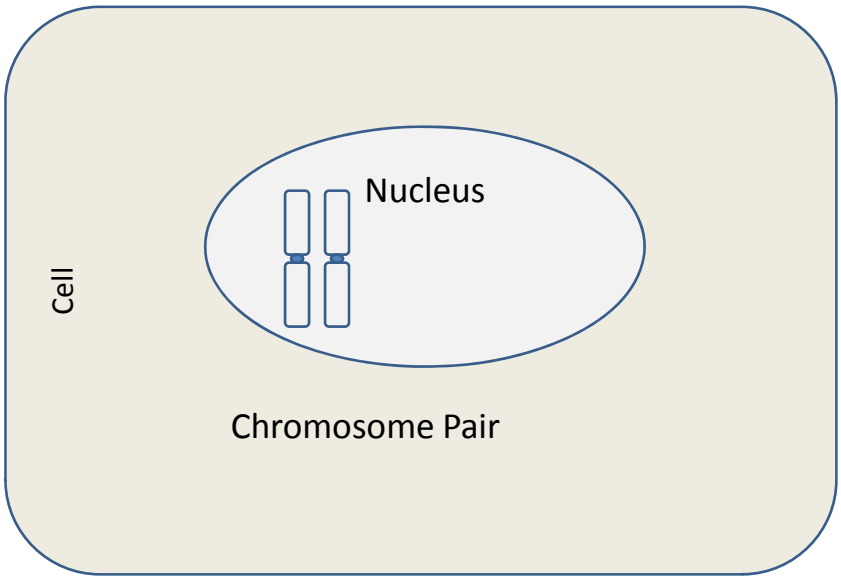
2. Pathways are introduced and the related gene controls are presented. The pathways which create the chemicals which in turn control cell growth and proliferation are discussed in some detail.

This discussion should provide the basic principles to address the other issue we seek to develop.

4.1.1 Chromosomes and Genes

Let is start with the chromosome. We will return in some detail to this latter but at this point we want to establish a few basic definitions. The human has 11 pairs of chromosomes, for a total of 23 chromosomes.

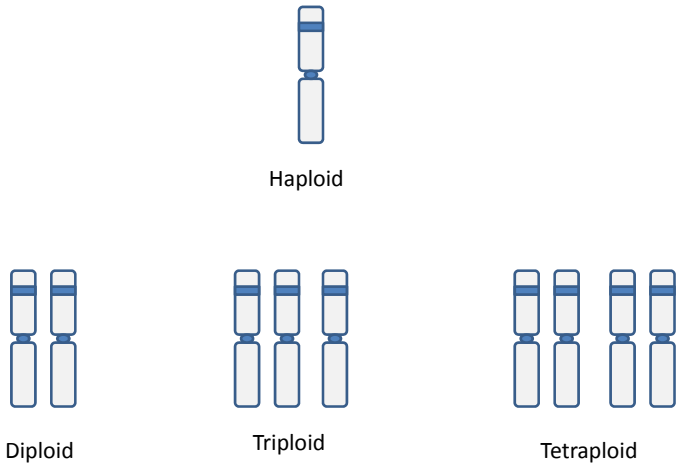
The Figure below is a graphic of a typical cell showing the nucleus and one of the chromosome pairs. This graphic is not at all what one would see in reality but it is typical of the generic elements.



4.1.2 Chromosome

The chromosomes are the collection of DNA which agglomerates together into separate units. They bind together as pairs and it is these pairs which make up the chromosomes we see in the nucleus of a mature cell.

The Figure below depicts the types of possible chromosome combinations we would. This is called ploidy, haploid being one chromosome and diploids being pairs of chromosomes.



The types of ploidy are:

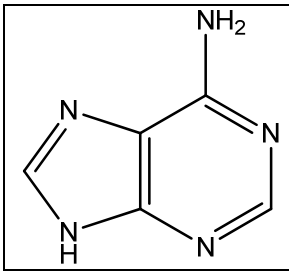
Haploid: The haploid is the single chromosome strand that one may be able to see in the sex cells. The haploid is a single stranded non-binded collection of DNA.

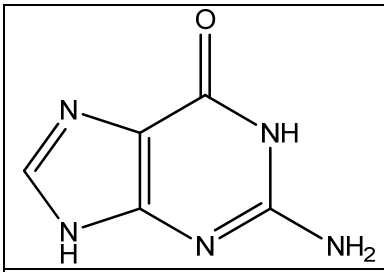
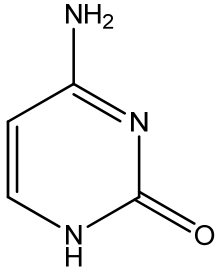
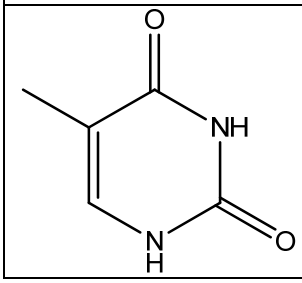
Diploid: The diploid is the prototypical collection of DNA. The diploid is merely two, one from the male and one from the female.

4.1.3 DNA

DNA, deoxyribonucleic acid is the heart of the gene. It is the basis of the code we can understand to determine the relationship between genes and their phenotypic responses.

We will now briefly layout the ideas concerning DNA in this section. DNA is constructed in the following manner. There are four base elements; Adenine (A), Guanine (G), Thymine (T) and Cytosine (C). They are shown below.

	Adenine

	Guanine
	Cytosine
	Thymine

These Base elements can combine in only a specific manner, namely A with T and G with C. These bonds are shown below. This was one of the seminal observations which drove Watson and Crick towards their great discovery. The bonding also is the basis for how these Bases combine in pairs, the Base Pairs, and then how these Base Pairs link up to form the now famous DNA chain.

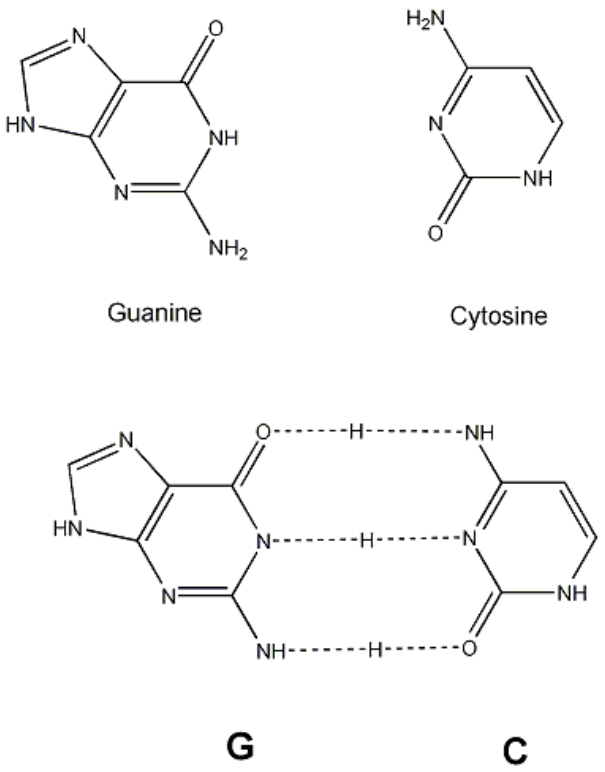


Figure 1 CT Base elements and their bonding.

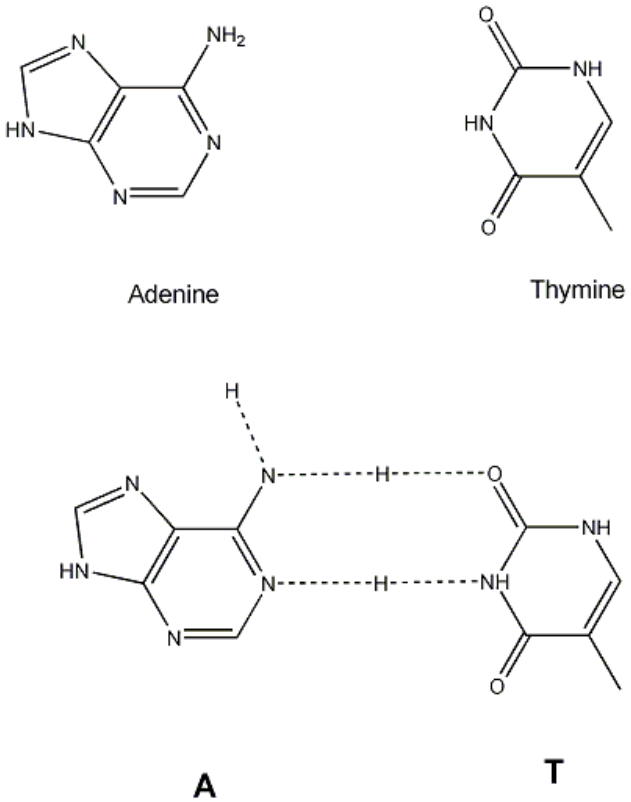
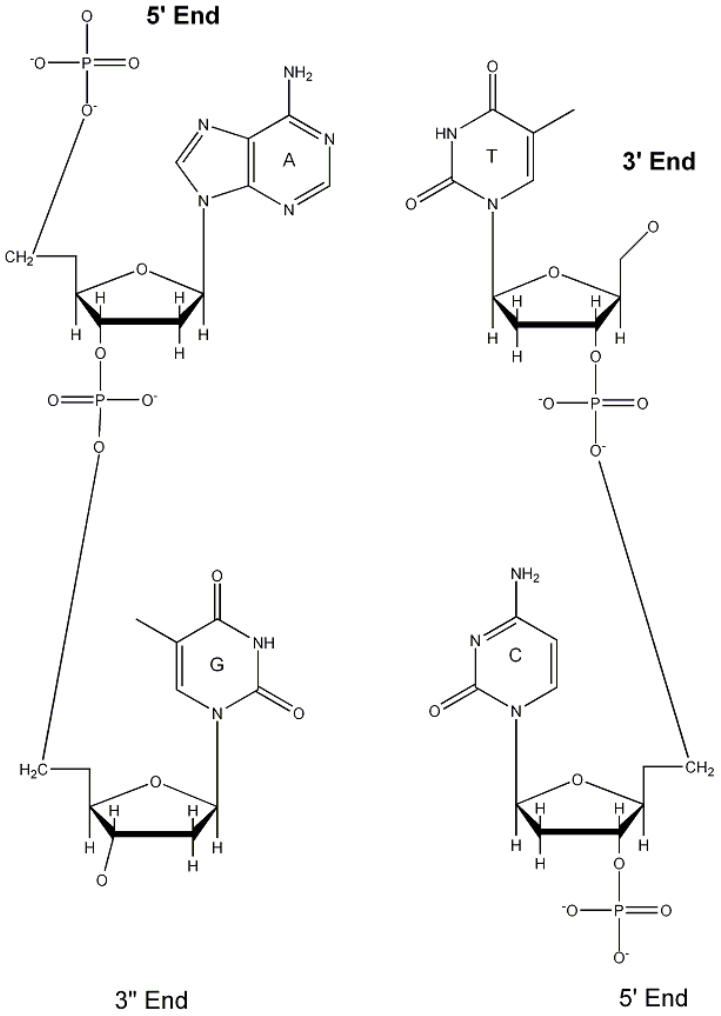


Figure 2 A-T Base Pairing

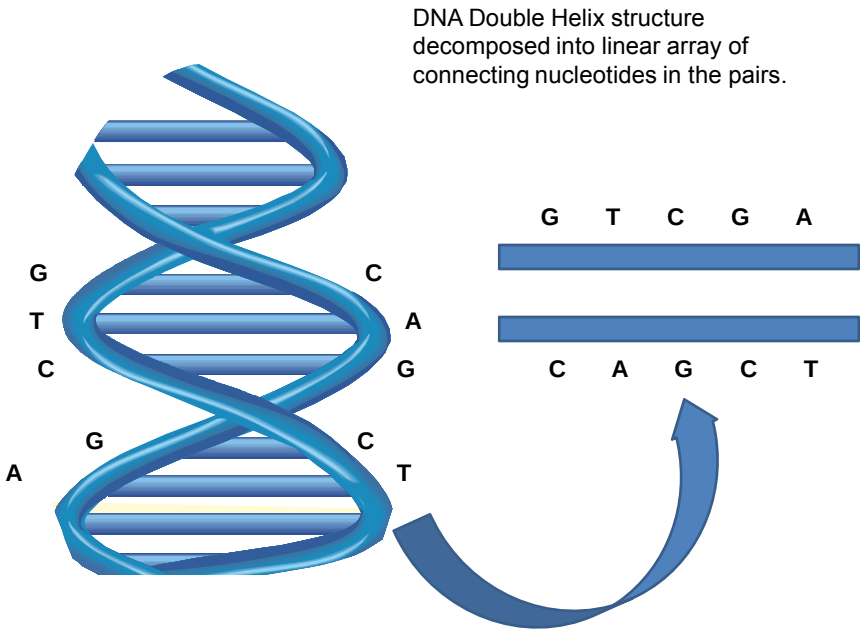
Now these Base Pairs are connected to sugar molecules, a cyclic ribose, to create a Nucleoside, such as deoxyadenosine. Then the nucleosides are enhanced with a phosphate constellation, a phosphorous molecules surrounded by oxygen and hydrogen. This combination of the nucleoside and the phosphate is called a Nucleotide. It is these nucleotides which connect on a backbone on the outside and in another backbone on the inside to form the DNA molecule.

The following Figure shows a Nucleotide connection, we do not show the base pair connections. The Nucleotide has two defined ends; a 3' end which of the OH molecule and the 5" end which is the phosphate. We show these in the following Figure. These ends will play an important part in the generation of the products of DNA.



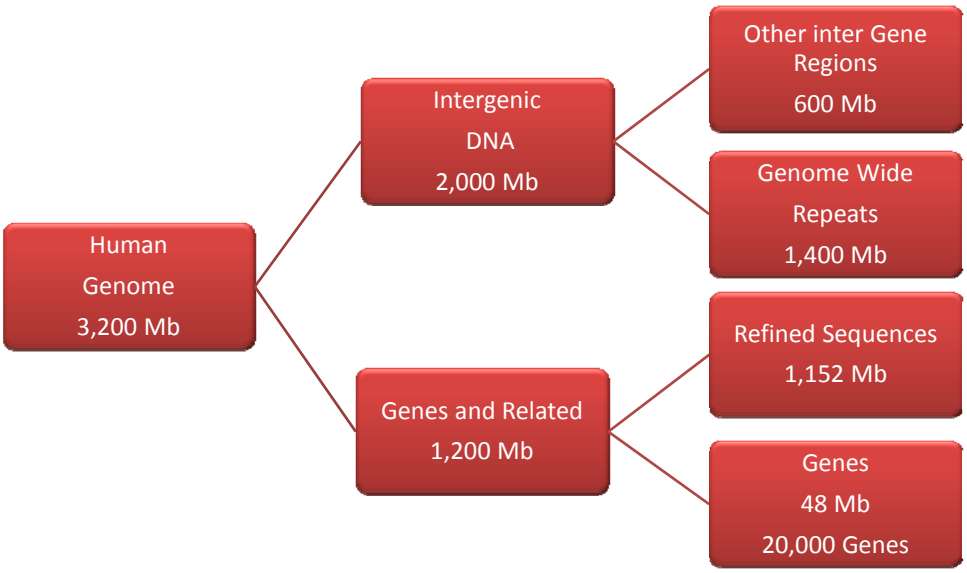
The nucleotides are then connected into the long DNA wrapped double helix which is generally well known. This is shown below. Our interest will be in the genes themselves and we will look at them in some detail. One of the key questions will be just what is a gene? That will be a challenging question. It will go to the heart of hybridizing. It can be answered in many ways but clearly the simple ideas of Mendel must be revisited.

In the Figure below we set forth a paradigm of the opposite bases and they are lined up in a stretched out set of nucleotides where we are looking solely at the base elements, the A, T, G and C.



In the human the DNA is of moderate size, about 3,200 Mb, that is 3.3 billion G, T, A, or C. However as shown below the DNA is broken down into many small elements. The actual operating genes constitute a mere 48 million bases and this constitute about 20,000 genes. That is an average of 2,400 bases per gene. As we shall see it takes three bases to create one chemical compound on a protein, this there are a total of 800 per protein on average.

The main conclusion is that there is a great deal of what has been called junk DNA. That DNA is useful for identifying people, namely that is used in DNA identification, and it may or may not play great roles in protein generation and gene modulation.



Ref: Watson et al, Molec. Bio Gene 5th Ed, p. 137

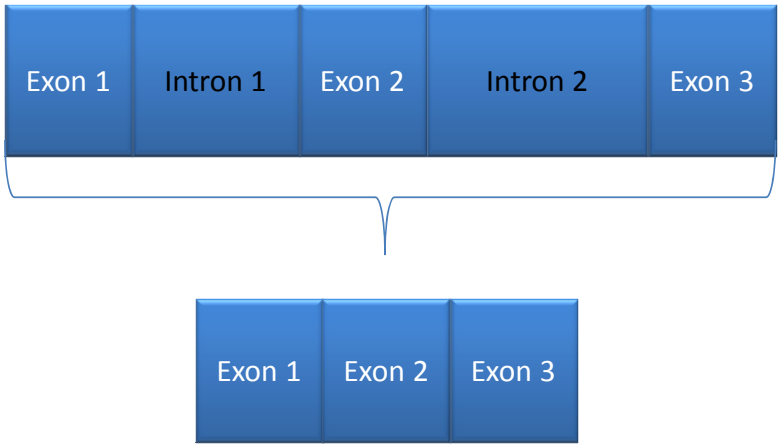
4.1.4 Gene

The gene is the fundamental building block of any living creature. It is not the single expressive element to control a phenotype, it may contribute to that control but it is not the one to one element in the process. Thus a red flower may be controlled by several genes and in addition those genes may be affected by several epigenetic factors ranging from the environment to other genes.

The human is now thought to have about 20,488 genes[35]. Not a large number and greatly lower than what literally all the experts thought before the human gene was fully analyzed. Many experts had guessed that there were well above 300,000 genes in the human. The Human genome is composed of slightly more than 3 Billion base pairs, combinations of G, T, C or A. The Hemerocallis genome is approximately 4 Billion base pairs. The number of active genes in Hemerocallis is at this time unknown. But it is close in size to the human genome.

The simple construct of a gene is shown below. It is a collection of DNA bases which combine together in terms of the effect. We show in the Figure the Introns, namely the unused DNA bases, and the exons, the used DNA bases. The exons are "combined" to effect what a gene does.

[35] See Pennisi, Working the (Gene Count) Numbers: Finally, a Firm Answer? SCIENCE Vol 316 25 May 2007 http://www.sciencemag.org/cgi/content/full/316/5828/1113a?maxtoshow=&HITS=10&hits=10&RESULTFORMAT=&fulltext=gene+count&searchid=1&FIRSTINDEX=0&resourcetype=HWCIT

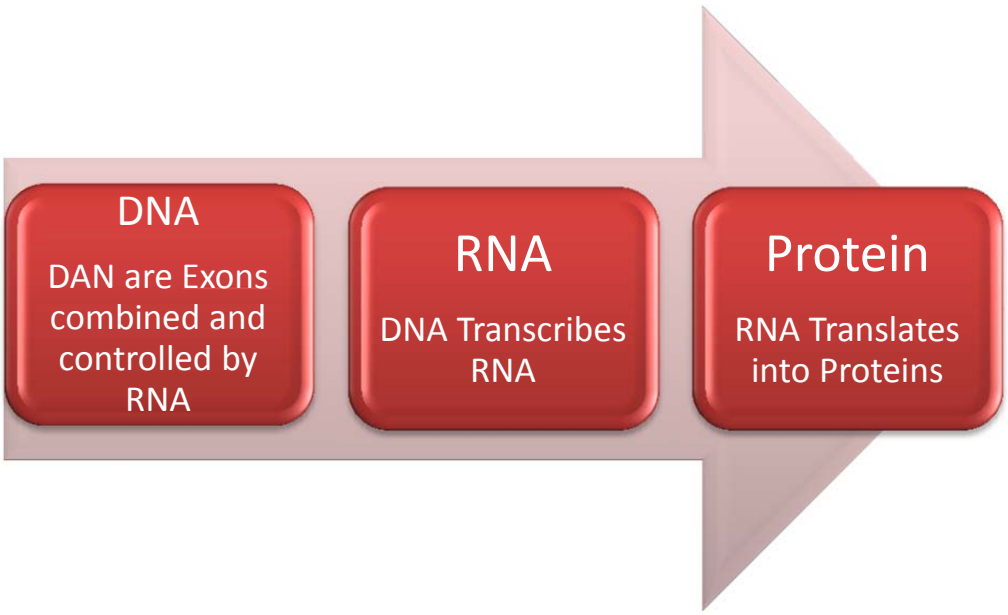


What then is a gene? For our purposes and to be consistent with contemporary understanding we define a gene as:

"A gene is a collection of DNA bases which when combined in a determinable manner can express the combination of bases via the production of some effect upon the cell and potentially its surrounding environment. A gene is an expressible collection of base pairs, when acting in concert, in the internal environment of a cell."

Thus we understand a gene by its effects, not just by its structure. It effects may be complex. It may produce some RNA, and in turn a protein, it may activate or suppress another gene, or it may be the basis for creating a new gene in this construct. Based upon what we know and understand today, a gene is not some well-defined coherent set of contiguous DNA. Genes can even be created on the fly within the cell based upon the environment that is if we define a gene by what it creates and affects.

The classic paradigm for DNA influence is shown below. Namely that DNA generates RNA via transcription and RNA generates proteins via translation. We will not get into further details other than saying that this process has many sub elements which will be regarded in further detail latter.



The above understanding of the gene and its relationship to its environment states that there exists a gene, a construct, which uniquely generate an RNA strand, which in turn uniquely generates a protein. We now know that these are all subject to further analysis. For example, the gene is not just a connected set of DNA bases, it is a set of exons, which may be combined in a sequence, or may even be broken or reassembles. Thus the gene is determined by what it does, not by any unique set of base pairs.

The protein that results from the above model is then related to some phenotypic response.

4.2 Genotype and Phenotype

Phenotypes are what we see, smell, hear, touch, taste; they are the interactions between some creatures.

Genotype is what the gene has as specific content, its specific DNA. The production of a phenotype is frequently driven by the expression of a gene. The gene "expresses" itself in a very special manner. The DNA is wrapped in tight coils.

The model we will build upon appears as in the Figure below. This is the canonical model for gene expression. We assume that there is some collection of secondary pathways, and that these pathways result in chemical products that are directly related to a phenotype; a darker red flower, a longer leaf, a taller scape. That these pathways are modulated in some manner by proteins generated from within a cell. That the proteins are the result of some entity called a gene. That the gene can be an assembly of bases and the gene may itself be modulated up or down by activator or repressor proteins respectively generated by other genes or even the same gene. Thus we model the cell as a dynamic system and further we argue that this system has certain random elements which we shall include latter.

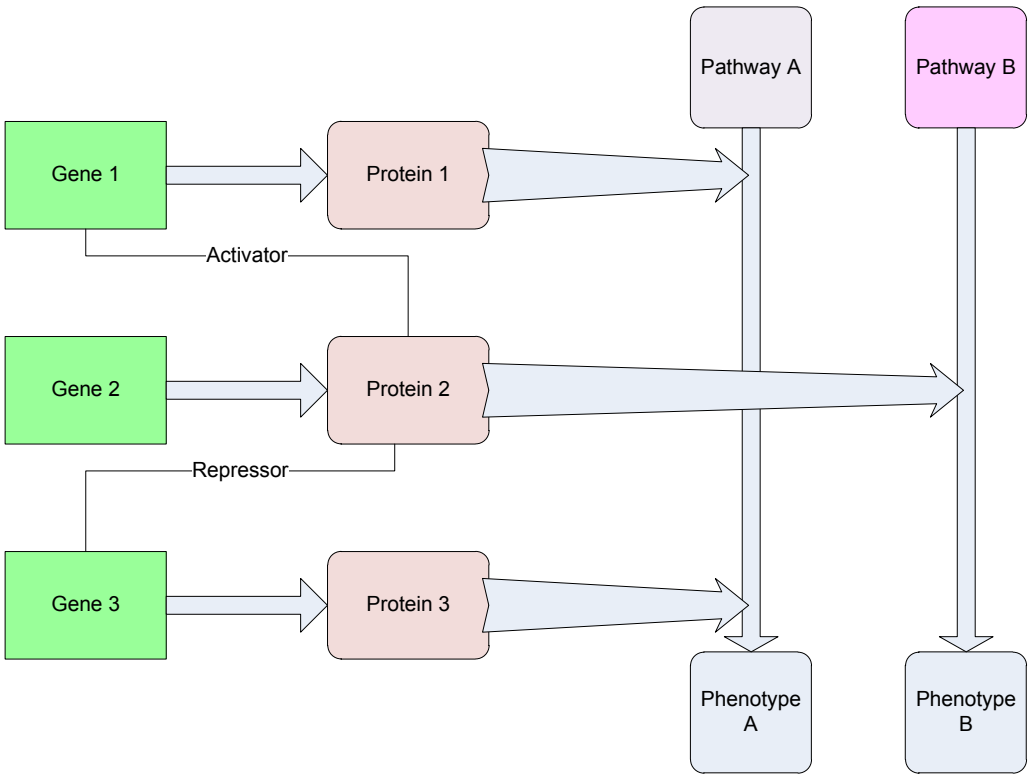


Figure Canonical Model for Gene Expression

It is the output of this genetic process that we get the full temporal and spatial existence.

The above model of the gene is one in which we see the beginnings of some form of feedback. We see the activator and repressor genes as the basis for this element. However this may be expanded even further, We show this below.

Note we show that the Gene K can be influenced by other Genes, as well as the products of the pathways as well as by the environment. The Environment can modulate the pathway which by being fed back to another controlling gene can then modulate the activating gene. This process is a complex process and exceeds what we would have imagined from the simple Mendellian gene theory.

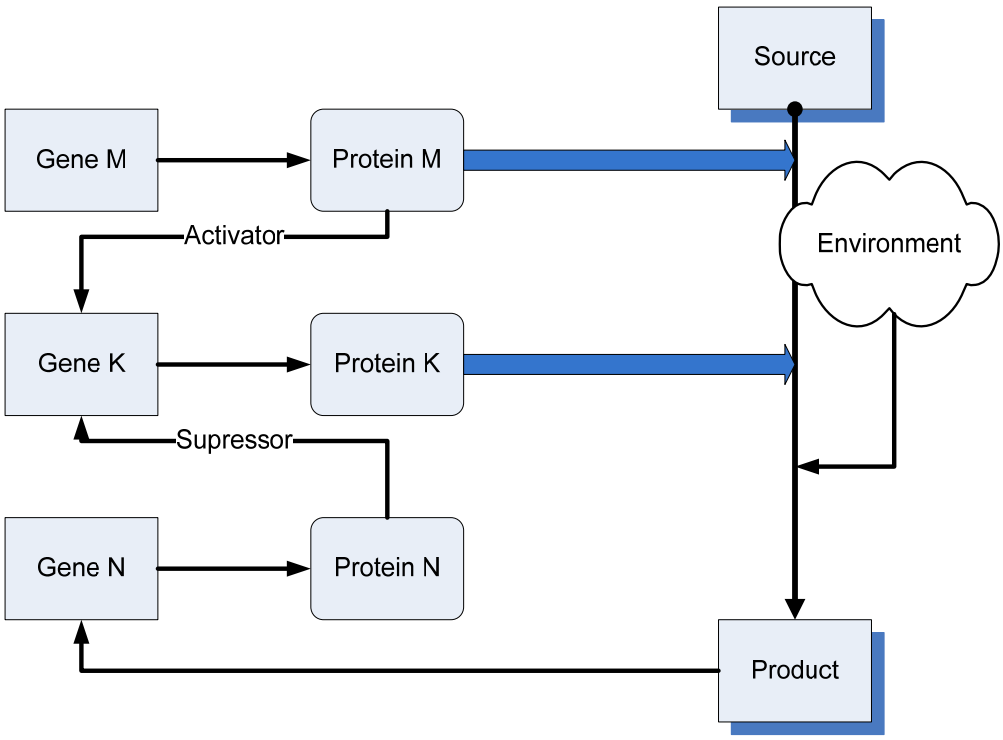


Figure Dynamic Gene Model

Now back one again to the Mendellian Gene model. Although Mendel and his model was not so rigidly simple, for he did admit some other influences as well as variation, we will call the simple Gene and Phenotype combination the Mendel Model. Namely in this model we assume the existence of a Gene and then we further assume that there is some phenotypic characteristic such as flower color which maps one to one onto this gene.

One gene and one phenotypic character. The phenotypic characters further have countable and discrete values. The flower is red, yellow, and green. There are no blends and there are a limited numbers. Then there is a gene for red, a gene for yellow and a gene for green. The gene is at the same place on the chromosome and the gene just somehow changes to produce a different color. In addition the genes are dominant in some order. That is if there is one red gene, of the two on the chromosome, then we get red, if not a red but a yellow we get yellow, and we get green if and only if there are all green genes, namely two.

Now there is a second model, based upon our understanding of DNA and the Watson Crick world. However this model goes well beyond the simple Watson Crick model. Here we assume we have long segments of DNA with many exons and many more introns. The gene as we know it is the result of the cellular processes which assemble the exons into a block of DNA which RNA will use to in turn generate a protein. In reality what happens is that the exons may be recombined to generate RNA in a variety of fashions. The result of that process, as well as the dynamic model we depicted above is that the phenotypic characteristic. It has a set of values whose probability distribution may be of some form. We use as an example a standard Gaussian curve. This is shown below.

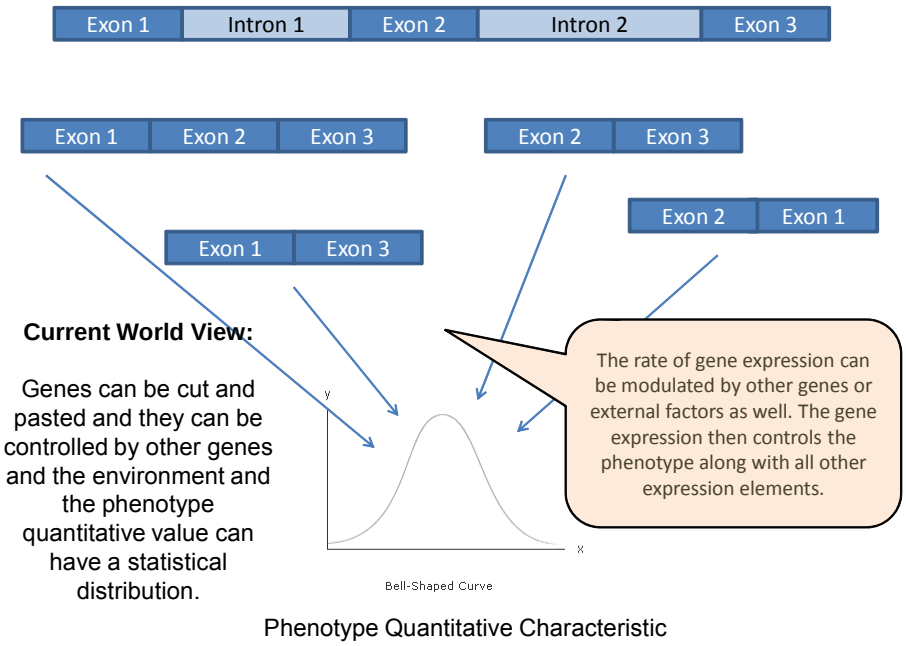
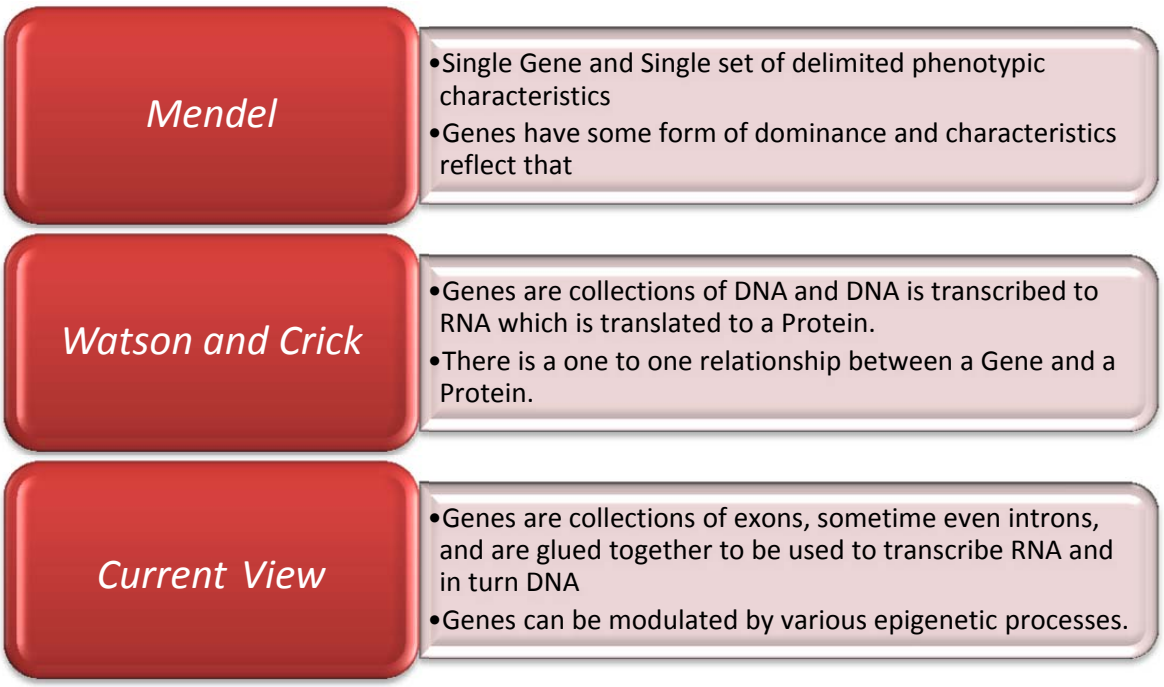


Figure Current View of Genetic Control



4.3 GENETICS

In this section we present an overview of the classic Mendellian analysis.[36] The Mendellian analysis makes classic assumptions which prevailed until the advent of the Watson and Crick model, and even slightly beyond. In fact many breeding programs build upon a Mendellian approach. We argue that such an approach is partially correct but lacks most of the key elements which must be considered.

In this section we briefly review the molecular genetics of a cell. We do not get into any significant details but merely review the elements which we can use letter in developing the mathematical models for regulation. The enzymes are proteins produced within the cell. The proteins are the result of the expression of a set of genes.

In this section we now by reviewing the current understanding of cell micro genetics show that the proteins are expressed by the normal process understood since Watson and Crick's seminal work and that there are factors which and activate their production, indeed enhance their production, or repress their production. These are the activators or repressor proteins. The activator and repressor proteins are in effect other genes expressing themselves.

What will be critical to understand here is that we just want to place the process of activators and repressors in context. We discuss in the next section what our overall design approach will be; that of an engineering model development and not a detailed understanding at the cell level. Frankly, we are not interested in the lower level detail, only gross modeling of cells, genes, and their proteins. They will become the inputs, outputs and control mechanisms of our design approach.

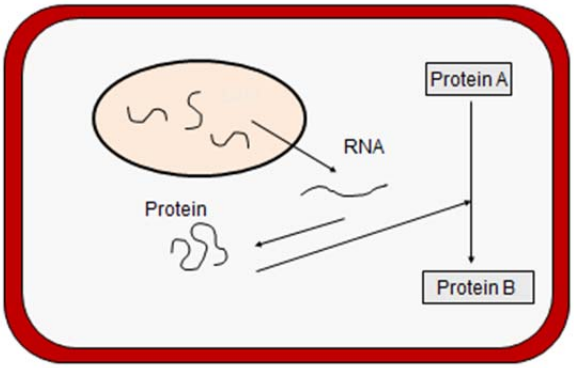
4.3.1 Cells and DNA

Cells of eukaryotic cells which are characterized primarily by having a flexible cell wall. The cells generate all of the amino acids they need for protein generation unlike animal cells but other than that, for our purposes, they function very much the same.

When we look at a collection of cells they appear as below. They are aligned and interconnect via various channels.

The graphic below summarizes the view we shall take. Each cell has DNA and the DNA uses a mRNA to create proteins. The proteins are then used in the management of the pathways to create the secondary products of the cell.

[36] See Griffiths. This is an excellent overview of genetic analysis.



We do however want to stress certain issues. There are two extreme views of cells:

Micro/Time View: The micro view looks at a cell at each instant of time and considers what is happening. Is the cell generating a protein and a secondary and if so how and what is the sequence in which this process occurs. It is a focus on a single cell over some time period and we see many things happening.

Ensemble View: In this case we look at the cell on average. Namely we say a cell can "on average" produce a protein and can then in turn produce a secondary.

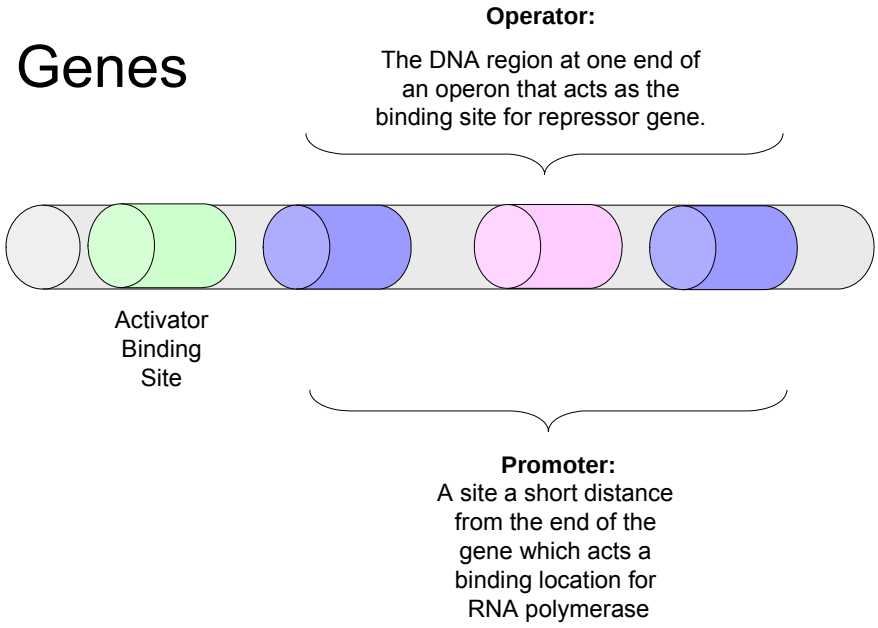
These two views have analogs in mathematical analysis; they are the time averages versus the ensemble average. In mathematical statistics we have the concept of looking at a single cell and time averaging say the concentration of a certain secondary. We know how it is produced and thus over some time window we can look at the average of say pelargonidin and we than measure its average value. In contrast we can take a collection of similar cells and measure the pelargonidin in each cell and take that average. The latter is called the ensemble average. The equivalence of the two is called the Ergodic Theorem and was developed by Norbert Wiener[37]. The microbiologist typically focuses on the time view. We in this Chapter will focus on the ensemble view. The latter view will allow us to model, predict and control large collections of cells.

4.3.2 Gene Processes

The processes in genes are generally identical to those in animal and thus human genes. The figure below shows a typical gene structure along with key sites. This structure shows the gene

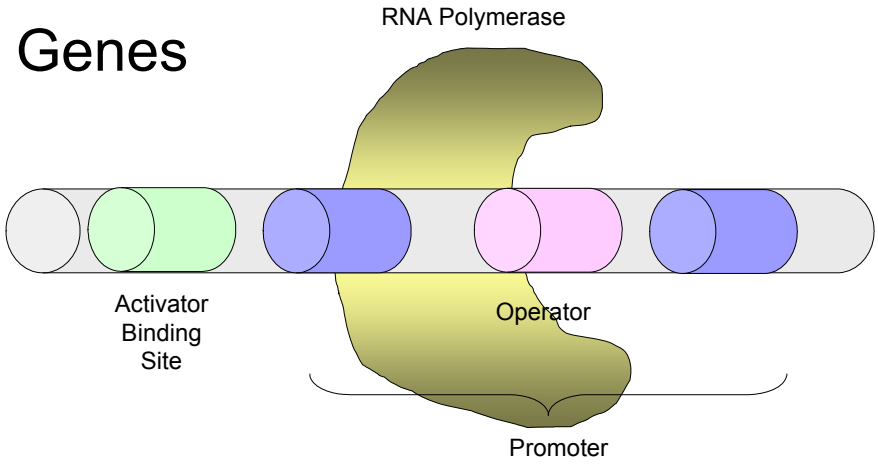
[37] See McGarty, Stochastic Systems and State Estimation.

activator site which is where activator proteins can bind to start or enhance the expression of the gene. The operator sits and the overall promoter sequence are shown down from the activator site.[38]



Genes express themselves with the assistance of RNA polymerase. The RNA polymerase is key in that it binds to the DNA and then opens it up to allow for the transcription creating the mRNA required for the translation process. In the figure below we show this process.

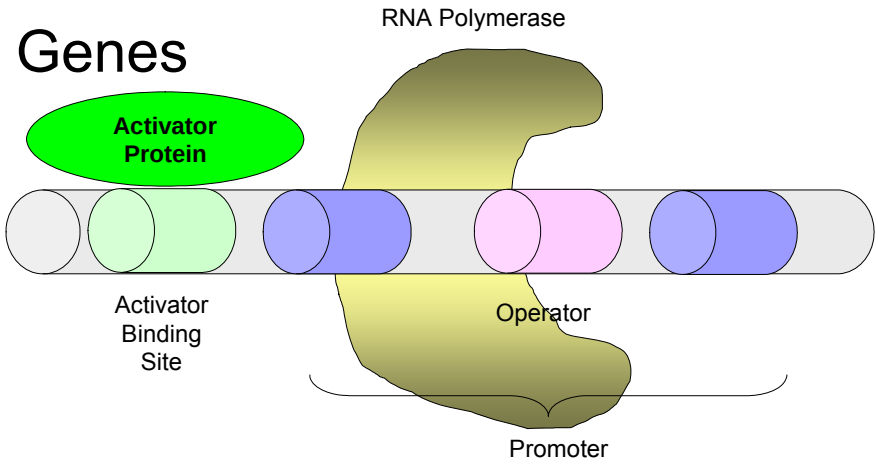
[38] This is detailed in Watson et al. Also see Griffiths et al.



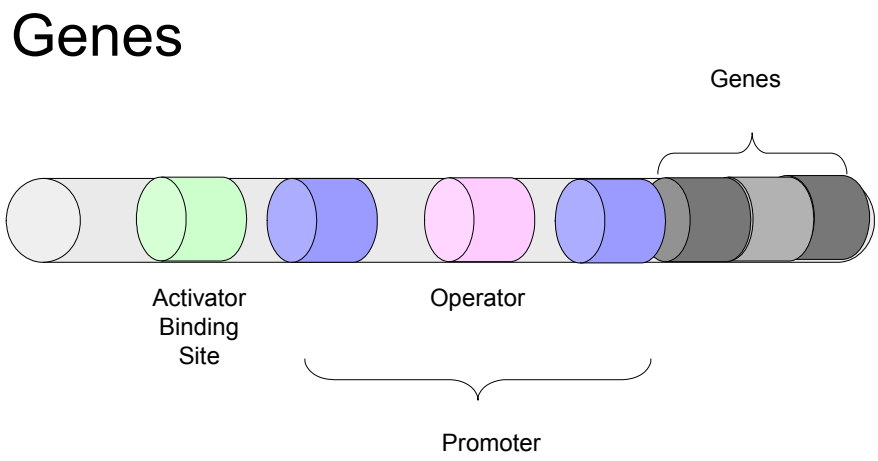
We will now focus on two actions which control the gene expression; activators and suppressors.

4.3.3 Activators

Activators are proteins which when attached to the gene assist in the expression of the gene. An activator is a protein resulting from another gene which can assist and facilitate the expression of a gene. Remember we want to look at the ensemble view, not the time view. Thus we assume that the RNA polymerase is continuously acting to produce proteins and that there is a continuous flow at some level of the activators. The cell process from the time view is shown below. An activator binds facilitates the RNA polymerase binding which in turn produces the mRNA and then in turn the proteins via the translation process.



If there is an activator then the gene can be readily expressed. The RNA polymerase then binds, creates the mRNA and this in turn produces the related protein. Activators stimulate this process. The Figure below depicts the location of the gene downstream from the activator and the promoter.



Now it is important to understand the activator from a time perspective and then from the ensemble perspective.

1. Activators are proteins generated by other genes in the cell.
2. Activators bind to the DNA and facilitate the production of the gene, which in turn produces another protein.
3. Activators can bind, release and then rebind. Each time they do that they produce another mRNA and that in turns produces another protein molecule.
4. From a time perspective, it is activator, produces gene reading, produces mRNA, and produces protein.
5. From an ensemble perspective we have a concentration of activator proteins and then we get a concentration of result proteins.

This then leads to a simple model:

$P_o = \text{Output Protein Concentration}$

$P_i = \text{Input Protein Concentration}$

$P_o = A_{o,i} P_i$

But there is also a dynamic model which we can state; to some degree this model is a hybrid of the time and ensemble approach. The model states:

$$\frac{dP_o}{dt} = f\left(P_o(t), P_i(t), t\right)$$
$$P_o(0) = P_o^0$$
$$P_i(0) = P_i^0$$

Now we must remember that this simple two protein, two gene model is just a simplification. In reality we may have dozens of not hundreds of genes in this process. Now consider a simple linear model for this two gene system:

$$P_i(t) = P_i^0 \exp(-\lambda_i t)$$

$$\frac{dP_o(t)}{dt} = A_{o,i} P_i(t) + A_{o,o} P_o(t)$$

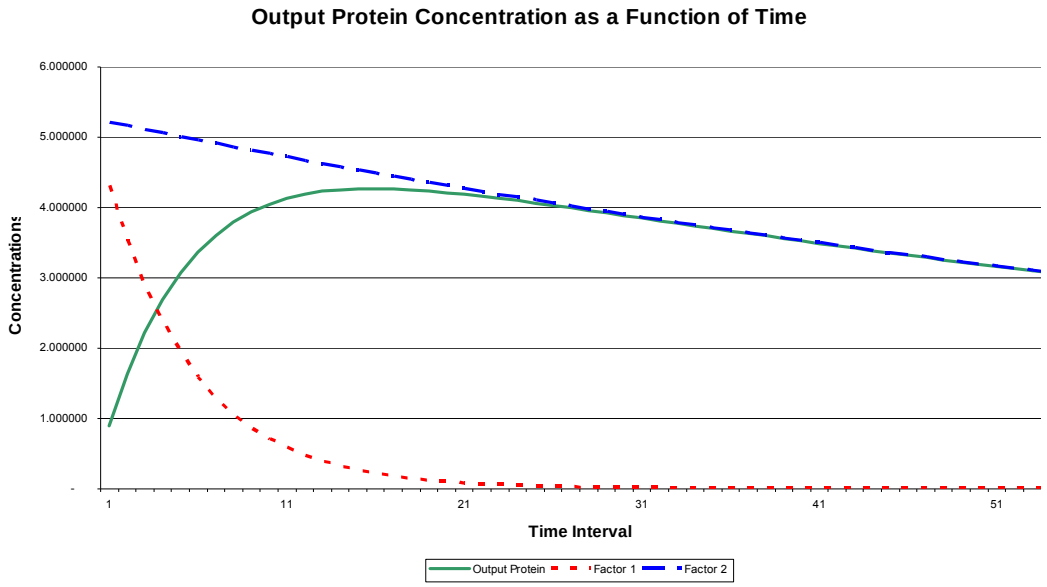
We can solve this differential equation. It is:

$$P_o(t) = k_{o,i} P_i(0) \left[\frac{\exp(-\lambda_i t) - \exp(-k_{o,o} t)}{\lambda_i - k_{o,o}} \right]$$

where;

$$A_{o,o} = -k_{o,o}$$
$$A_{o,i} = +k_{o,i}$$

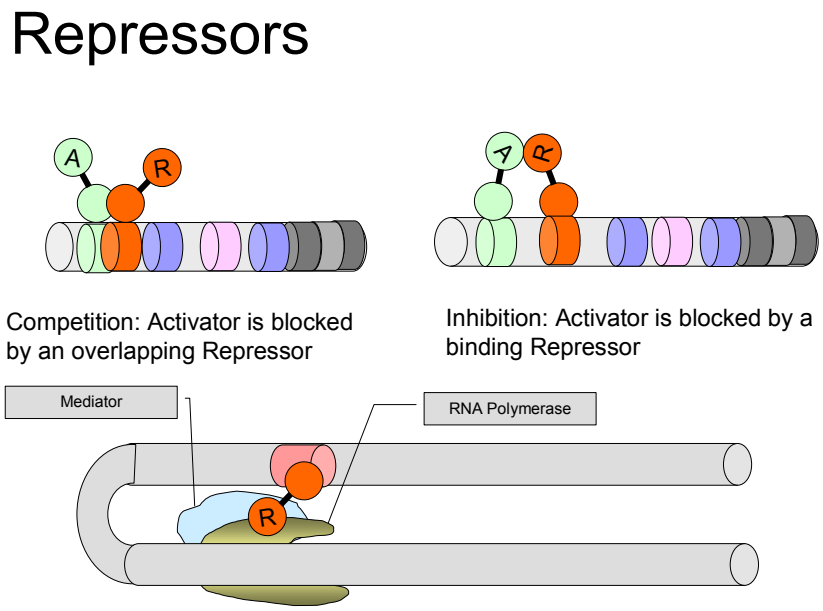
We have solved this for a simple example using constants of 0.01 and 0.2 respectively.



Note that the output protein concentration reaches a peak and then decays as per the driving protein. We will see this phenomenon again.

4.3.4 Repressors

In contrast to activators we also have genes which are suppressors. Three methods of suppressor action are shown below. A suppressor does the opposite of an activator. It suppresses the expression of a gene. The same logic will follow the repressor as was with activators. We again also want to view this from an ensemble perspective.



As we did with the activator, we see a repressor stops the generation of the protein. This it is nothing more than a negative driver to protein generation.

4.4 Expression Analysis and Implications

In this section we develop a systems approach to the problem of color analysis and synthesis. This work is based upon the recent work of Szallasi and others. However this also builds upon the work in McGarty (1971) which focused a systems approach to the overall identification problem.

4.4.1 Approach: Engineering versus Science

The approach we take in this Chapter is an engineering approach rather than a biological approach.[39] Our interest is in developing a model or sets of models which allow us by a verifiable means to show how the genes react and interact. We can compare this to the engineering approach to circuit design of transistor circuits versus the science of understanding the semiconductor from the point of view of detailed quantum mechanical models.

The biologist in our approach is akin to the physicists and engineers who approach the cell from the bottom up, trying to understand all of the intricate processes and steps that lead at the micro level to the developments we look at herein. In our approach it is akin to the engineer knowing

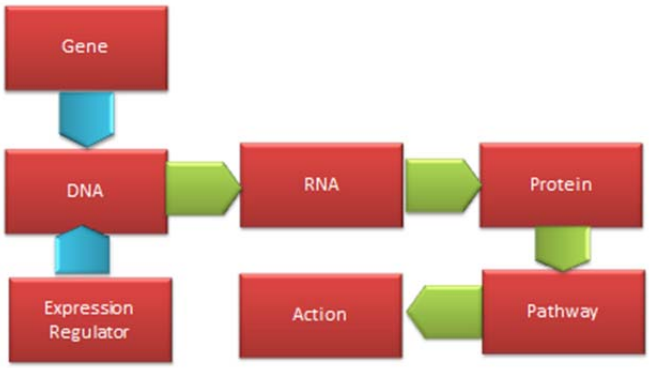
[39] There has been a significant set of development recently in analyzing genetic data from a systems perspective. In this Chapter we have taken such an approach. The recent work by such authors as Perkins et al, Vohradsky, Hatzimanikatis et al, and the recent book by Szallasi are seminal. However, there is an issue here also or world view and what does one really want from the analysis. The bench scientist looks to understand all the details of the underlying processes. The engineer seeks to understand enough to model the process and to do so with a reasonable degree of accuracy but the ultimate goal for the engineer is control of the process and generation of new processes.

that there is some function inside the semiconductors which may clearly be important but the engineer's interest is in designing and analyzing the transistor as a circuit element.

Thus for an engineer, if we increase a current here we get a decrease or an increase at some other point. The engineer creates a world view of a macro set of processes and models the details of the biologists in our case with a few set of equations which show the results of increases and decreases. This model must then be valid table and verifiable. One must be able to make measurements to show that the processes predicted indeed occur, to a reasonable degree of accuracy. Then one can analyze a genetic circuit and then in addition one can design a genetic circuit. We then can understand where the colors come from and possibly engineer the genes to develop and deliver on colors we desire.

4.4.2 A Control Paradigm

The basic control paradigm is contained in the following Figure. The expression regulator may be an activator or suppressor. It may be a result of a gene expression in the cell itself or quite possibly as we shall discuss fed through from another cell. There are many of these regulatory cycles and they are all interconnected. This basic paradigm is one of hundreds or thousands of such interconnected flows.



In developing our models we will use this construct. However, we can frequently focus on natural clusters of related genes. They may be a dozen or more such related genes in each cluster and possibly hundred of such clusters. Although cells and their proteins may affect all other cells, only a few of the genes regulated have a significant level of regulation. The low levels of "regulation" we shall consider just as noise.

4.5 CELL REPLICATION

We first address cell replication. First we examine the cell cycle from a generic perspective. We then examine the details on the pathways which may result in unstable cell reproduction.

4.5.1 Cell Cycle

The cell replication cycle goes through 4 stages. The dormant stage, G0, is not part of this process. The stages in cell reproduction are:

G0: This is the resting phase. It is during this phase that the cell is producing proteins via normal transcription processes. G0 may be resting related to the reproductive mitotic activities but the cell is quite active as a protein generating factory.

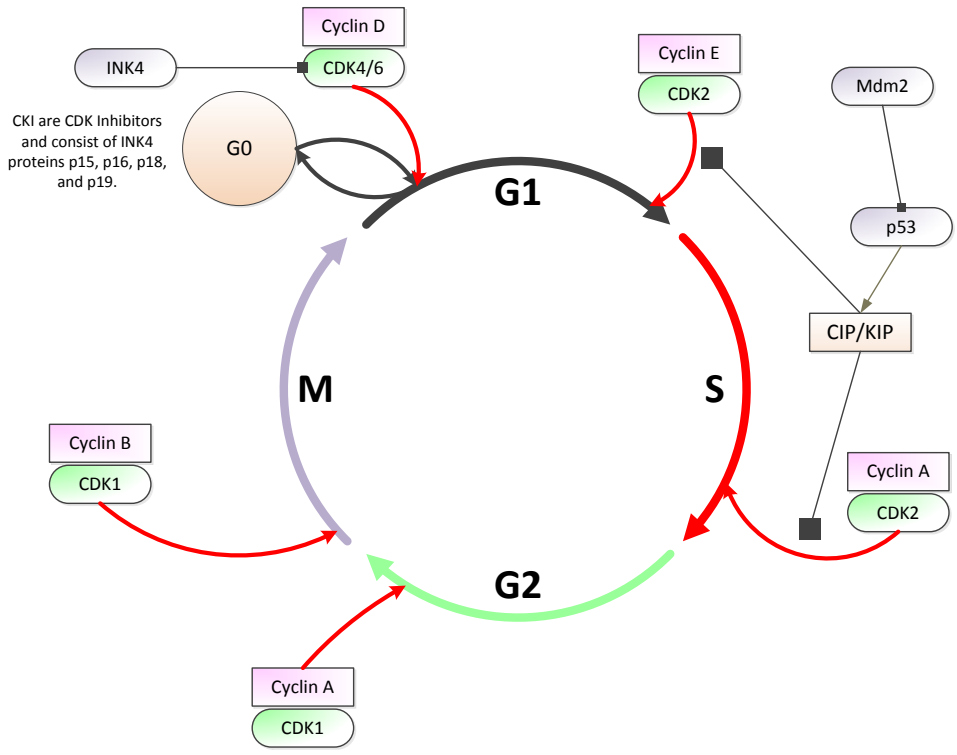
G1: Once the cell begins the G1 phase it is on its way to reproducing via mitosis.

S: The S phase is the phase where the DNA is duplicated. This is a sensitive stage, any error here can be propagated forward albeit there may still be checks available.

G2: This is the second gap phase.

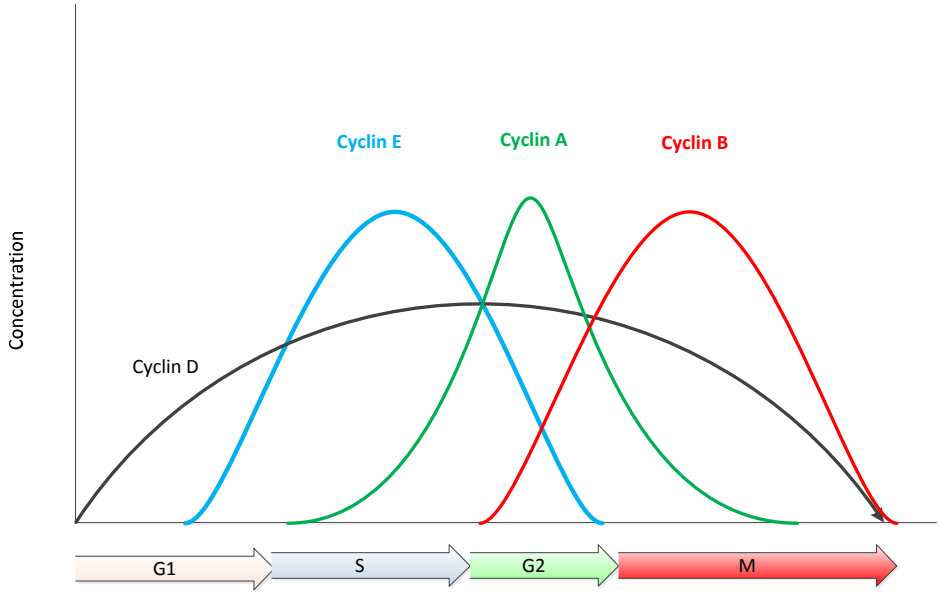
M: M phase includes mitosis and cytokinesis, namely the creation of two identical new cells.

Now the cell starts G1 by being instigated by a bound pair of a cyclin and a CDK, a cyclin dependent kinase. In this specific case we start with a binding of cyclin D and CDK4/6. This is the initiating event moving into G1 from senescence in G0. We depict these processes below (from McKinnell et al p. 169.):



The cyclins in each stage grow in concentration and as such move the cell along in each of its reproductive stages.

The following shows the phases and the relevant concentrations of cyclin bound to CDKs. Note the increase in concentration activates a change or movement along the mitotic path.



Note in the above the concentration of a specific cyclin above a level of a previous cyclin initiates the next step in mitosis. The details as to how and why this happens is detailed in Morgan (Chapter 3).

Protein[40]	***Gene***	***Function***[41]
Cyclin A (also CCN1; CCNA, CCNA2, Cyclin A2)	4q25-q31	The protein encoded by this gene belongs to the highly conserved cyclin family, whose members are characterized by a dramatic periodicity in protein abundance through the cell cycle. Cyclins function as regulators of CDK kinases. Different cyclins exhibit distinct expression and degradation patterns which contribute to the temporal coordination of each mitotic event. In contrast to cyclin A1, which is present only in germ cells, this cyclin is expressed in all tissues tested. This cyclin binds and activates CDC2 or CDK2 kinases, and thus promotes both cell cycle G1/S and G2/M transitions.

[40] http://www.ncbi.nlm.nih.gov/gene/983

[41] From http://www.ncbi.nlm.nih.gov/gene/595 data bases as a source.

Protein[40]	*Gene*	*Function*[41]
Cyclin B1 (CCNB1)	5q12	The protein encoded by this gene is a regulatory protein involved in mitosis. The gene product complexes with p34(cdc2) to form the maturation-promoting factor (MPF). Two alternative transcripts have been found, a constitutively expressed transcript and a cell cycle-regulated transcript, that is expressed predominantly during G2/M phase. The different transcripts result from the use of alternate transcription initiation sites.
Cyclin B2 (CCNB2)	15q22.2	Cyclin B2 is a member of the cyclin family, specifically the B-type cyclins. The B-type cyclins, B1 and B2, associate with p34cdc2 and are essential components of the cell cycle regulatory machinery. B1 and B2 differ in their subcellular localization. Cyclin B1 co-localizes with microtubules, whereas cyclin B2 is primarily associated with the Golgi region. Cyclin B2 also binds to transforming growth factor beta RII and thus cyclin B2/cdc2 may play a key role in transforming growth factor beta-mediated cell cycle control.
Cyclin C (CCNC)	6q21	The protein encoded by this gene is a member of the cyclin family of proteins. The encoded protein interacts with cyclin-dependent kinase 8 and induces the phophorylation of the carboxy-terminal domain of the large subunit of RNA polymerase II. The level of mRNAs for this gene peaks in the G1 phase of the cell cycle. Two transcript variants encoding different isoforms have been found for this gene.
Cyclin D (Cyclin D1)	11q13	The protein encoded by this gene belongs to the highly conserved cyclin family, whose members are characterized by a dramatic periodicity in protein abundance throughout the cell cycle. Cyclins function as regulators of CDK kinases. Different cyclins exhibit distinct expression and degradation patterns which contribute to the temporal coordination of each mitotic event. This cyclin forms a complex with and functions as a regulatory subunit of CDK4 or CDK6, whose activity is required for cell cycle G1/S transition. This protein has been shown to interact with tumor suppressor protein Rb and the expression of this gene is regulated positively by Rb. Mutations, amplification and overexpression of this gene, which alters cell cycle progression, are observed frequently in a variety of tumors and may contribute to tumorigenesis.

Protein[40]	*Gene*	*Function*[41]
Cyclin E (CCNE1)[42]	19q12	The protein encoded by this gene belongs to the highly conserved cyclin family, whose members are characterized by a dramatic periodicity in protein abundance through the cell cycle. Cyclins function as regulators of CDK kinases. Different cyclins exhibit distinct expression and degradation patterns which contribute to the temporal coordination of each mitotic event. This cyclin forms a complex with and functions as a regulatory subunit of CDK2, whose activity is required for cell cycle G1/S transition. This protein accumulates at the G1-S phase boundary and is degraded as cells progress through S phase. Overexpression of this gene has been observed in many tumors, which results in chromosome instability, and thus may contribute to tumorigenesis. This protein was found to associate with, and be involved in, the phosphorylation of NPAT protein (nuclear protein mapped to the ATM locus), which participates in cell-cycle regulated histone gene expression and plays a critical role in promoting cell-cycle progression in the absence of pRB. Two alternatively spliced transcript variants of this gene, which encode distinct isoforms, have been described.

The CDKs involved are:

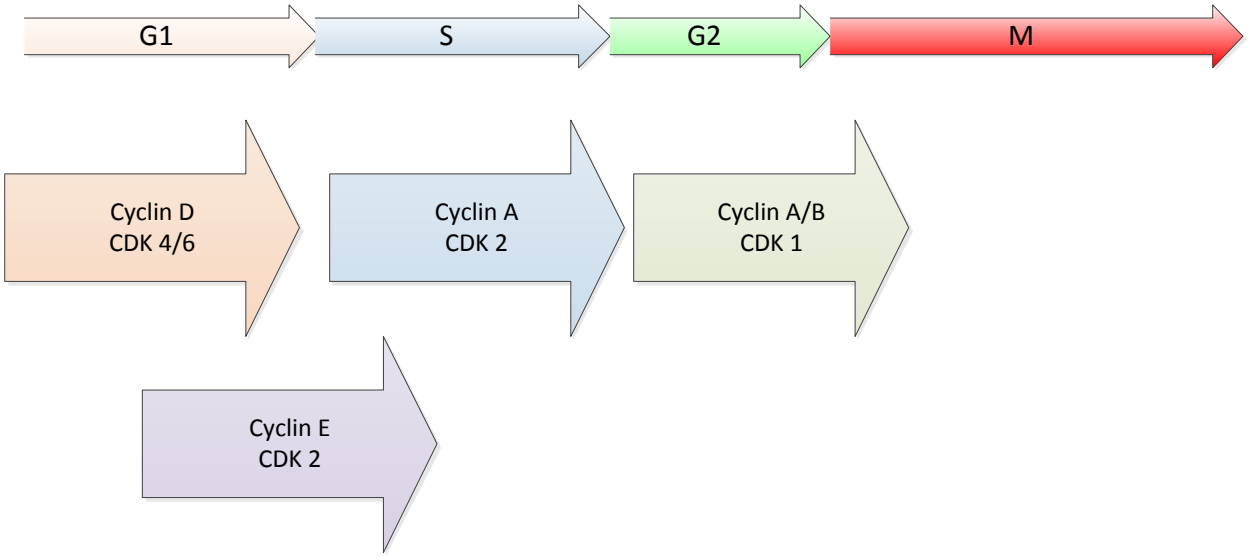
[42] http://www.ncbi.nlm.nih.gov/gene/898

Protein[43]	*Gene*	*Function*[44]
CDK 1 (also known as CDC2; CDC28A; P34CDC2)	10q21.1	This protein is a catalytic subunit of the highly conserved protein kinase complex known as M-phase promoting factor (MPF), which is essential for G1/S and G2/M phase transitions of eukaryotic cell cycle. Mitotic cyclins stably associate with this protein and function as regulatory subunits. The kinase activity of this protein is controlled by cyclin accumulation and destruction through the cell cycle. The phosphorylation and dephosphorylation of this protein also play important regulatory roles in cell cycle control.
CDK 2 (also called p33)	12q13	It is a catalytic subunit of the cyclin-dependent protein kinase complex, whose activity is restricted to the G1-S phase, and essential for cell cycle G1/S phase transition. This protein associates with and regulated by the regulatory subunits of the complex including cyclin A or E, CDK inhibitor p21Cip1 (CDKN1A) and p27Kip1 (CDKN1B). Its activity is also regulated by its protein phosphorylation.
CDK 3	17q22	This gene encodes a member of the cyclin-dependent protein kinase family. The protein promotes entry into S phase, in part by activating members of the E2F family of transcription factors. The protein also associates with cyclin C and phosphorylates the retinoblastoma 1 protein to promote exit from G0.
CDK 4 (also CMM3; PSK-J3)	12q14	This protein is a catalytic subunit of the protein kinase complex that is important for cell cycle G1 phase progression. The activity of this kinase is restricted to the G1-S phase, which is controlled by the regulatory subunits D-type cyclins and CDK inhibitor p16(INK4a). This kinase was shown to be responsible for the phosphorylation of retinoblastoma gene product (Rb). Mutations in this gene as well as in its related proteins including D-type cyclins, p16(INK4a) and Rb were all found to be associated with tumorigenesis of a variety of cancers.
CDK 6 (also PLSTIRE)	7q21-22	The protein encoded by this gene is a member of the cyclin-dependent protein kinase (CDK) family. CDK family members are known to be important regulators of cell cycle progression. This kinase is a catalytic subunit of the protein kinase complex that is important for cell cycle G1 phase progression and G1/S transition. The activity of this k+inase first appears in mid-G1 phase, which is controlled by the regulatory subunits including D-type cyclins and members of INK4 family of CDK inhibitors. This kinase, as well as CDK4, has been shown to phosphorylate, and thus regulate the activity of, tumor suppressor protein Rb. Expression of this gene is up-regulated in some types of cancer.

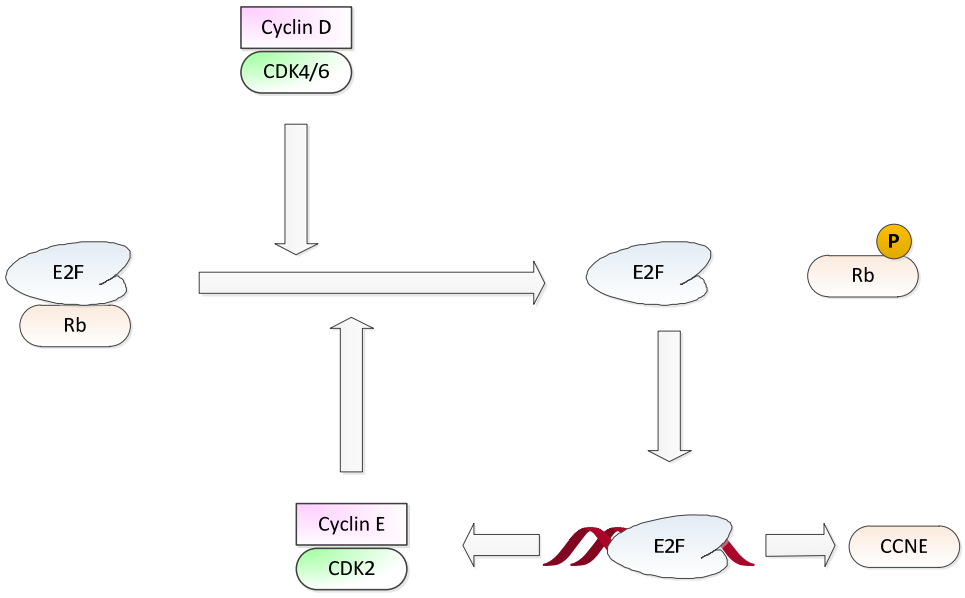
[43] http://www.ncbi.nlm.nih.gov/gene/983

[44] From http://www.ncbi.nlm.nih.gov/gene/595 data bases as a source.

Now the question is what activates these proteins, the cyclins and the CDKs, to make the cell cycle progress. This begins the creep upward in this pathway concern. We can redraw this process as follows and it will help to focus:



Now we ask what activates these proteins. We look at the activation of Cyclin E as shown by Bunz (p 219) below:



This is a feedback type reaction initiated by Rb the retinoblastoma gene protein. This feedback generates cyclin E which drives the cell through G1 and into the S cycle.

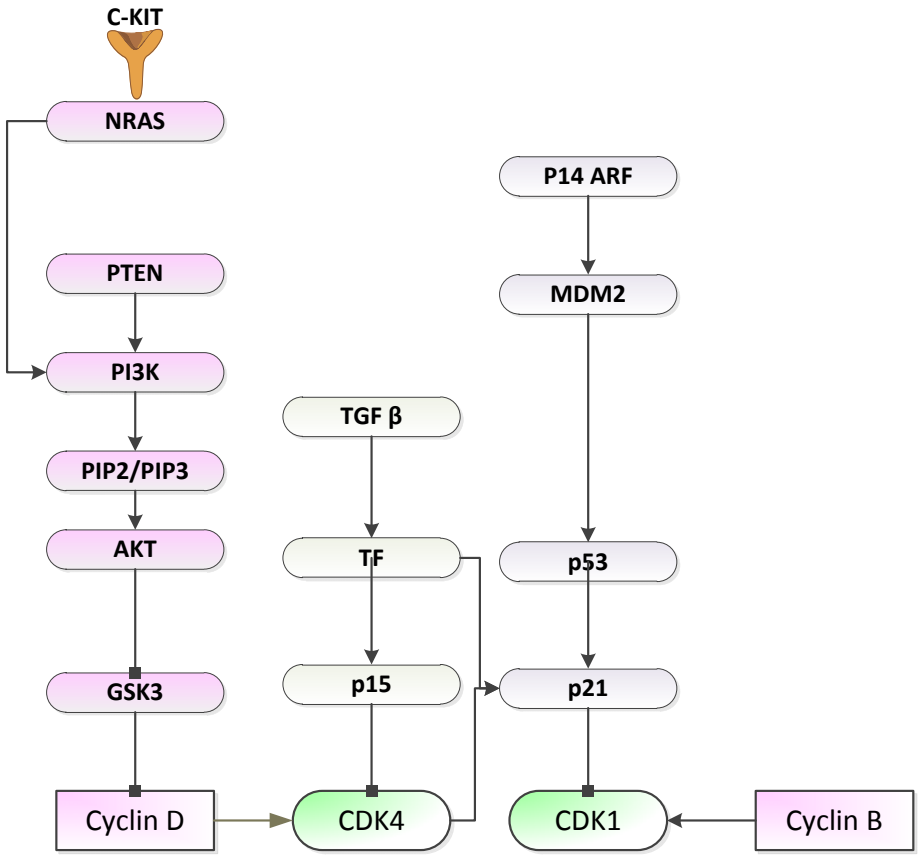
Gene	*Location*	*Function*
E2F1[45] (also RBP3; E2F-1; RBAP1; RBBP3)	20q11.2	The protein encoded by this gene is a member of the E2F family of transcription factors. The E2F family plays a crucial role in the control of cell cycle and action of tumor suppressor proteins and is also a target of the transforming proteins of small DNA tumor viruses. The E2F proteins contain several evolutionally conserved domains found in most members of the family. These domains include a DNA binding domain, a dimerization domain which determines interaction with the differentiation regulated transcription factor proteins (DP), a transactivation domain enriched in acidic amino acids, and a tumor suppressor protein association domain which is embedded within the transactivation domain. This protein and another 2 members, E2F2 and E2F3, have an additional cyclin binding domain. This protein binds preferentially to retinoblastoma protein pRB in a cell-cycle dependent manner. It can mediate both cell proliferation and p53-dependent/independent apoptosis.
RB 1[46] (also RB; pRb; OSRC; pp110; p105-Rb)	13q14.2	The protein encoded by this gene is a negative regulator of the cell cycle and was the first tumor suppressor gene found. The encoded protein also stabilizes constitutive heterochromatin to maintain the overall chromatin structure. The active, hypophosphorylated form of the protein binds transcription factor E2F1. Defects in this gene are a cause of childhood cancer retinoblastoma (RB), bladder cancer, and osteogenic sarcoma.
CCNE1[47]	19q12	The protein encoded by this gene belongs to the highly conserved cyclin family, whose members are characterized by a dramatic periodicity in protein abundance through the cell cycle. Cyclins function as regulators of CDK kinases. Different cyclins exhibit distinct expression and degradation patterns which contribute to the temporal coordination of each mitotic event. This cyclin forms a complex with and functions as a regulatory subunit of CDK2, whose activity is required for cell cycle G1/S transition. This protein accumulates at the G1-S phase boundary and is degraded as cells progress through S phase. Overexpression of this gene has been observed in many tumors, which results in chromosome instability, and thus may contribute to tumorigenesis. This protein was found to associate with, and be involved in, the phosphorylation of NPAT protein (nuclear protein mapped to the ATM locus), which participates in cell-cycle regulated histone gene expression and plays a critical role in promoting cell-cycle progression in the absence of pRB. Two alternatively spliced transcript variants of this gene, which encode distinct isoforms, have been described.

[45] http://www.ncbi.nlm.nih.gov/gene/1869

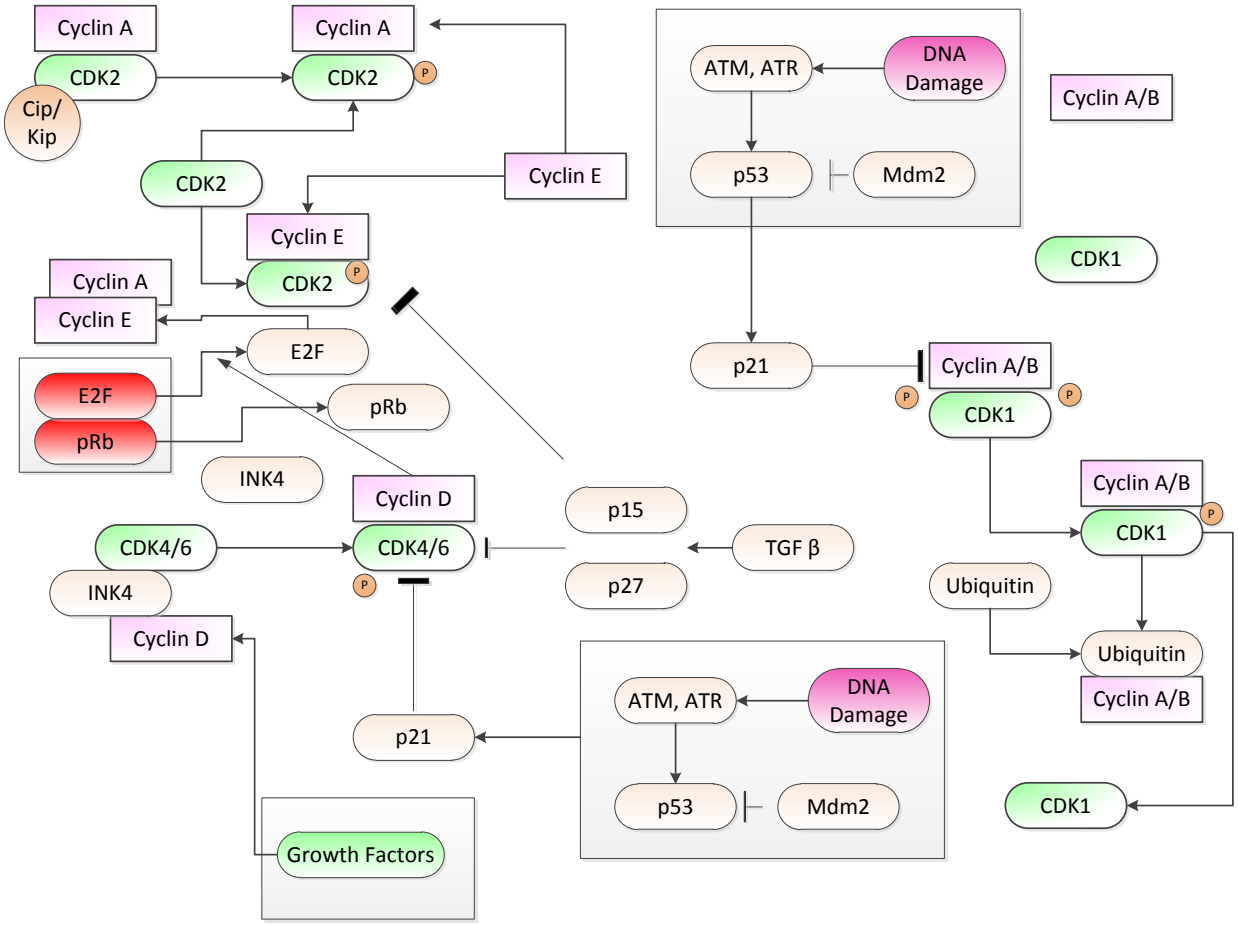
[46] http://www.ncbi.nlm.nih.gov/gene/5925

[47] http://www.ncbi.nlm.nih.gov/gene/898

Now this establishes one base line for understanding cancer at the base of cell reproduction. Namely what can cause this process to continue unabated?



A more details analysis has been by Vermulen et al almost a decade ago. We shall use this as a baseline and then add to what we have learned in that period. The Vermulen network is shown as follows:



Now in the Vermulen configuration we have the following elements:

1. CDKs: These are the cyclin dependent kinases we have been discussing.

2. Cyclins:

3. CDK Activating Enzymes:

4. CKI or CK Inhibitors

The following is a detailed list of some major CKIs or Cyclin Kinase Inhibitors. We have discussed them briefly before but they play a critical role in managing cell reproduction.

CKI Family	*Member Name*	*Alternative Name*	*Gene*	*Function*
INK4 Family	p15[48] (also P15; MTS2; TP15; CDK4I; INK4B; p15INK4b)	INK-4b	9p21	This gene lies adjacent to the tumor suppressor gene CDKN2A in a region that is frequently mutated and deleted in a wide variety of tumors. This gene encodes a cyclin-dependent kinase inhibitor, which forms a complex with CDK4 or CDK6, and prevents the activation of the CDK kinases, thus the encoded protein functions as a cell growth regulator that controls cell cycle G1 progression. The expression of this gene was found to be dramatically induced by TGF beta, which suggested its role in the TGF beta induced growth inhibition.
	p16[49] (also ARF; MLM; P14; P16; P19; CMM2; INK4; MTS1; TP16; CDK4I; CDKN2; INK4A; MTS-1; P14ARF; P19ARF; P16INK4; P16INK4A; P16-INK4A)	INK-4a	9p21	This gene generates several transcript variants which differ in their first exons. At least three alternatively spliced variants encoding distinct proteins have been reported, two of which encode structurally related isoforms known to function as inhibitors of CDK4 kinase. The remaining transcript includes an alternate first exon located 20 Kb upstream of the remainder of the gene; this transcript contains an alternate open reading frame (ARF) that specifies a protein which is structurally unrelated to the products of the other variants. This ARF product functions as a stabilizer of the tumor suppressor protein p53 as it can interact with, and sequester, MDM1, a protein responsible for the degradation of p53. In spite of the structural and functional differences, the CDK inhibitor isoforms and the ARF product encoded by this gene, through the regulatory roles of CDK4 and p53 in cell cycle G1 progression, share a common functionality in cell cycle G1 control.

[48] http://www.ncbi.nlm.nih.gov/gene/1030

[49] http://www.ncbi.nlm.nih.gov/gene/1029

CKI Family	*Member Name*	*Alternative Name*	*Gene*	*Function*
	p18[50]	INK-4c	1p32	The protein encoded by this gene is a member of the INK4 family of cyclin-dependent kinase inhibitors. This protein has been shown to interact with CDK4 or CDK6, and prevent the activation of the CDK kinases, thus function as a cell growth regulator that controls cell cycle G1 progression. Ectopic expression of this gene was shown to suppress the growth of human cells in a manner that appears to correlate with the presence of a wild-type RB1 function. Studies in the knockout mice suggested the roles of this gene in regulating spermatogenesis, as well as in suppressing tumorigenesis.
	p19[51]	INK-4d	19p13	The protein encoded by this gene is a member of the INK4 family of cyclin-dependent kinase inhibitors. This protein has been shown to form a stable complex with CDK4 or CDK6, and prevent the activation of the CDK kinases, thus function as a cell growth regulator that controls cell cycle G1 progression. The abundance of the transcript of this gene was found to oscillate in a cell-cycle dependent manner with the lowest expression at mid G1 and a maximal expression during S phase. The negative regulation of the cell cycle involved in this protein was shown to participate in repressing neuronal proliferation, as well as spermatogenesis.

[50] http://www.ncbi.nlm.nih.gov/gene/1031

[51] http://www.ncbi.nlm.nih.gov/gene/1032

CKI Family	*Member Name*	*Alternative Name*	*Gene*	*Function*
Cip-Kip Family	p21[52] also P21; CIP1; SDI1; WAF1; CAP20; CDKN1; MDA-6; p21CIP1	Waf1, Cip1	6p21.2	This gene encodes a potent cyclin-dependent kinase inhibitor. The encoded protein binds to and inhibits the activity of cyclin-CDK2 or -CDK4 complexes, and thus functions as a regulator of cell cycle progression at G1. The expression of this gene is tightly controlled by the tumor suppressor protein p53, through which this protein mediates the p53-dependent cell cycle G1 phase arrest in response to a variety of stress stimuli. This protein can interact with proliferating cell nuclear antigen (PCNA), a DNA polymerase accessory factor, and plays a regulatory role in S phase DNA replication and DNA damage repair. This protein was reported to be specifically cleaved by CASP3-like caspases, which thus leads to a dramatic activation of CDK2, and may be instrumental in the execution of apoptosis following caspase activation.
	p27[53] also p27; Rpn4	Cip2	12q24.31-q24.32	The 26S proteasome is a multicatalytic proteinase complex with a highly ordered structure composed of 2 complexes, a 20S core and a 19S regulator. The 20S core is composed of 4 rings of 28 non-identical subunits; 2 rings are composed of 7 alpha subunits and 2 rings are composed of 7 beta subunits. The 19S regulator is composed of a base, which contains 6 ATPase subunits and 2 non-ATPase subunits, and a lid, which contains up to 10 non-ATPase subunits. Proteasomes are distributed throughout eukaryotic cells at a high concentration and cleave peptides in an ATP/ubiquitin-dependent process in a non-lysosomal pathway. An essential function of a modified proteasome, the immunoproteasome, is the processing of class I MHC peptides. This gene encodes a non-ATPase subunit of the 19S regulator.

[52] http://www.ncbi.nlm.nih.gov/gene/1026

[53] http://www.ncbi.nlm.nih.gov/gene/5715

CKI Family	*Member Name*	*Alternative Name*	*Gene*	*Function*
	p57[54] also BWS; WBS; p57; BWCR; KIP2	Kip2	11p15.5	This gene is imprinted, with preferential expression of the maternal allele. The encoded protein is a tight-binding, strong inhibitor of several G1 cyclin/Cdk complexes and a negative regulator of cell proliferation. Mutations in this gene are implicated in sporadic cancers and Beckwith-Wiedemann syndorome, suggesting that this gene is a tumor suppressor candidate.

5. Substrates:

6. Checkpoint Proteins:

4.5.2 Cell Cycle Control

The following depicts cell cycle control:

Gene	*Location*	*Function*
Jun[55]	1p32-p31	This gene is the putative transforming gene of avian sarcoma virus 17. It encodes a protein which is highly similar to the viral protein, and which interacts directly with specific target DNA sequences to regulate gene expression. This gene is intronless and is mapped to 1p32-p31, a chromosomal region involved in both translocations and deletions in human malignancies.
Fos[56]	14q24.3	The Fos gene family consists of 4 members: FOS, FOSB, FOSL1, and FOSL2. These genes encode leucine zipper proteins that can dimerize with proteins of the JUN family, thereby forming the transcription factor complex AP-1. As such, the FOS proteins have been implicated as regulators of cell proliferation, differentiation, and transformation. In some cases, expression of the FOS gene has also been associated with apoptotic cell death.

[54] http://www.ncbi.nlm.nih.gov/gene/1028

[55] http://www.ncbi.nlm.nih.gov/gene/3725

[56] http://www.ncbi.nlm.nih.gov/gene/2353

Gene	*Location*	*Function*
Myc[57]	8q24.21	The protein encoded by this gene is a multifunctional, nuclear phosphoprotein that plays a role in cell cycle progression, apoptosis and cellular transformation. It functions as a transcription factor that regulates transcription of specific target genes. Mutations, overexpression, rearrangement and translocation of this gene have been associated with a variety of hematopoietic tumors, leukemias and lymphomas, including Burkitt lymphoma. There is evidence to show that alternative translation initiations from an upstream, in-frame non-AUG (CUG) and a downstream AUG start site result in the production of two isoforms with distinct N-termini. The synthesis of non-AUG initiated protein is suppressed in Burkitt's lymphomas, suggesting its importance in the normal function of this gene

4.5.3 Kinetics of Cell Cycles

One of the questions we may ask is related to the kinetics of these processes. For example in many cancers the cell doubling time is highly variable at different locations and at different times and with different cells.

4.6 Other Factors in the Cell Cycle

In a recent paper by Solimini et al the authors discuss the concepts of STOP and GO genes and carcinogenesis[58]. The paper reports on some extensive experimental results focusing on the issue of proliferation and the loss of certain sets of gene sites, the STP and GO sites.

The authors begin by discussing the current concepts of changes in oncogenes and tumor suppressor genes, some of the key pathway elements that we examine in analyzing intracellular pathway dynamics. They state:

Cancer progression is directed by alterations in oncogenes and tumor suppressor genes (TSGs) that provide a competitive advantage to increase proliferation, survival, and metastasis. The cancer genome is riddled with amplifications, deletions, rearrangements, point mutations, loss of heterozygosity (LOH), and epigenetic changes that collectively result in tumorigenesis.

How these changes contribute to the disease is a central question in cancer biology. In his "two-hit hypothesis," Knudson proposed that two mutations in the same gene are required for tumorigenesis, indicating a recessive disease. In addition, there are now several examples of haploinsufficient TSGs .

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[57] http://www.ncbi.nlm.nih.gov/gene/4609

[58] Solimini, N., et al, Recurrent Hemizygous Deletions in Cancers May Optimize Proliferative Potential, Science, 6 JULY 2012 VOL 337, p 104.

Current models do not explain the recent observation that hemizygous recurrent deletions are found in most tumors. Whether multiple genes within such regions contribute to the tumorigenic phenotype remains to be elucidated…

The last sentence regarding the inability to explain the presence of hemizygous deletions under the current model is the main driver for this effort. Thus they argue and demonstrate experimentally that:

Tumors exhibit numerous recurrent hemizygous focal deletions that contain no known tumor suppressors and are poorly understood. To investigate whether these regions contribute to tumorigenesis, we searched genetically for genes with cancer-relevant properties within these hemizygous deletions.

We identified STOP and GO genes, which negatively and positively regulate proliferation, respectively.

STOP genes include many known tumor suppressors, whereas GO genes are enriched for essential genes.

Analysis of their chromosomal distribution revealed that recurring deletions preferentially over-represent STOP genes and under-represent GO genes.

*We propose a hypothesis called the **cancer gene island model**, whereby gene islands encompassing high densities of STOP genes and low densities of GO genes are hemizygously deleted to maximize proliferative fitness through cumulative haploinsufficiencies.*

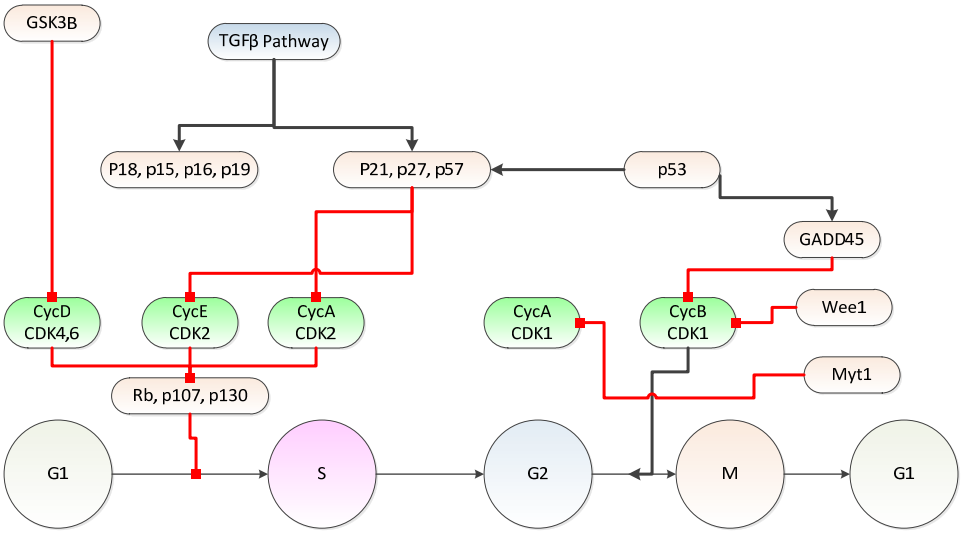
Because hundreds to thousands of genes are hemizygously deleted per tumor, this mechanism may help to drive tumorigenesis across many cancer types.

This is an intriguing hypothesis. It adds more pieces to an already complex puzzle. The Cancer Gene Island, CGI, hypothesis seems to indicate the complex changes in multiple gene sites. In particular there was a deletion of the STOP genes in preference to the GO genes. Unfortunately there did not seem to be a mechanism for these deletions, however the experimental evidence does indicate the phenomenon.

In their experimental analysis they have observed certain in vitro results which compel their hypothesis. They state:

This in silico analysis suggests that the loss of a single copy of GO genes has a negative impact on cellular fitness. To independently test this hypothesis, we turned to the other arm of our screen that identified candidate GO genes whose depletion limits proliferation and survival. Because both normal and cancer cells are dependent on these essential GO genes, we analyzed data from proliferation screens on HMECs, one normal prostate epithelial cell line, and seven breast or prostate cancer cell lines

They provide an interesting pathway model as shown below (as mofified, and also not that they have short hairpin RNAs (shRNAs)).



From Solimini et al: *"Known pathways with multiple shRNAs in the validation screen. Genes that validated in the secondary screen by a factor of ≥4 with multiple shRNAs are denoted with circles corresponding to three to five shRNAs (orange) and six or more shRNAs (red)."*

They conclude as follows:

The enrichment for genes localized to deletions suggests that we have identified dozens of new TSGs in recurrent deletions. We have also likely identified more TSGs outside of these regions because the STOP gene set is (i) enriched for known TSGs, many of which are not found in recurrent deletions, and (ii) enriched for genes that undergo somatic loss-of-function mutation.

Finally, this work suggests that cells possess a substantial number of genes that restrain proliferation in vitro, which could be inactivated to promote clonal expansion during tumorigenesis in addition to the traditional driver genes currently known. Given the prevalence of multiple, large, recurring hemizygous deletions encompassing skewed distributions of growth control genes in tumors, we propose that the elimination of cancer gene islands that optimize fitness through cumulative haplo-insufficiencies may play an important role in driving tumorigenesis, with implications for the way in which we think about cancer evolution.

As with many such works this raises as many questions as it seems to answer. However the control or lack thereof of proliferation and the cell cycle is a critical issue in carcinogenesis.

The following is from Ugurel et al[59]

Gene	*Action*
Melanocyte Lineage/Differentiation Antigens	
gp100 / HMB45	increased expression
Tumor Suppressors/Oncogenes/Signal Transducers	
AP-2 (activator protein-2 alpha) transcription factor	loss of nuclear AP-2 expression
bcl-6	expression
c-Kit	expression
c-met	expression
c-myc	increased expression
CYLD	decreased expression
EGFR (epidermal growth factor receptor)	increased expression
ERK (extracellular signal-regulated kinase)	absence of cytoplasmic ERK activation
HER3	increased expression
HDM2 (human homologue of murine mdm2)	increased expression
ING3	decreased nuclear expression
MITF (microphthalmia-associated transcription factor)	gene amplification
$p16^{INK4A}$	decreased expression
p-Akt (activated serine-threonine protein kinase B)	increased expression
pRb (retinoblastoma protein)	inactivation due to protein phosphorylation
PTEN	decreased expression
Cell Cycle Associated Proteins	
cyclin A, B, D, E	increased expression
geminin	increased expression
Ki67 (detected by Mib1)	increased expression
$p21^{CIP1}$	decreased expression
PCNA (proliferating cell nuclear antigen)	increased expression
Regulators of Apoptosis	
APAF-1 (apoptotic protease activating factor-1)	decreased expression
bak	decreased expression
bax	decreased expression
bcl-2	increased expression
survivin	increased expression
Molecules Involved in Angiogenesis	
LYVE-1 (lymphatic vascular endothelial hyaluronan receptor-1)	increased expression
PTN (pleiotrophin)	increased expression
Molecules Involved in Cell Adhesion and Motility	
beta-catenin	loss of nuclear staining
CEACAM1 (carcinoembryonic antigen-related cell-adhesion molecule 1)	increased expression
dysadherin	increased expression
E-cadherin	decreased expression
integrins beta-1 and beta-3	increased expression
MMPs (matrix metalloproteinases)	increased expression

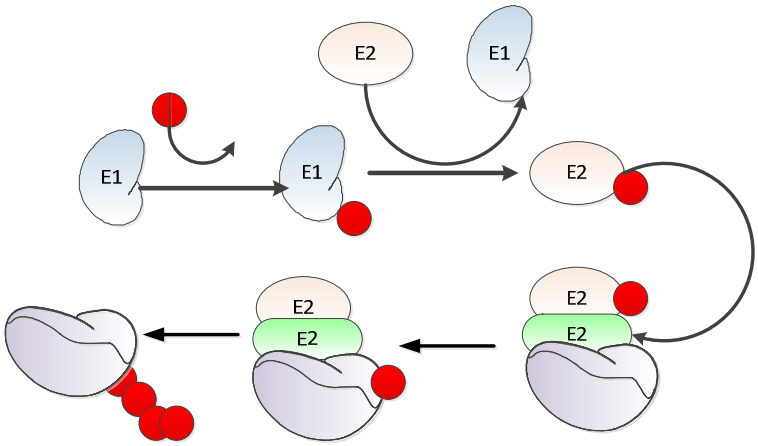
[59] Ugurel, S. et al Tumor Biomarkers in Melanoma, July 2009, Vol. 16, No. 3, Cancer Control, p. 219.

Gene	*Action*
osteonectin (also termed BM40 or SPARC [secreted protein, acidic and rich in cysteine])	increased expression
P-cadherin	strong cytoplasmic expression
Immunoregulators	
HLA allele frequency	specific expression
Others	
ALCAM/CD166 (Activated leukocyte cell adhesion molecule)	increased expression
CXCR4 receptor	increased expression
melastatin	decreased expression
metallothionein	increased expression
osteopontin	increased expression
TA (telomerase activity)	increased expression

As we have indicated, cell stability also includes the ability of a cell to recognize where it is and remaining in that location relative to other cells. One of the first signs of malignancy is a cell failing to understand its place and setting out on its own. In melanoma one of these very first steps is called melanoma in situ.

4.7 UBIQUINATION

Ubiquitin is a small protein which acts with three related proteins; E1, E2, and E3. E1 is also called the ubiquitin activating enzyme, E2 the ubiquitin conjugating enzyme, and E3 ubiquitin ligase. Together they act to attach ubiquitin to a target protein and mark it for digestion and elimination. The process is shown below in general graphic form.



Ubiquination is an essential process within a cell to eliminate used or excess proteins. Although we will not discuss tis in detail, it is an essential process and the reader should refer to standard texts[60].

[60] See: Cassimeris et al p 688, Weinberg, p 242, Alberts et al, p 1065.

4.8 TELOMERES AND MELANOMA

Telomeres are those ends of DNA which have the tendency to be lost each time a cell reproduces leading eventually to a loss of function. Cancer on the other hand may have mastered the loss of sections of the telomeres and thus may have an ability to prolong their life to many reproductions, namely unlimited. There has been significant interest in targeting telomeres and especially the related enzyme, telomerase, to control cancer cells. In a recent pair of papers the authors have focused on this process in melanomas and especially on UV activation.

The authors have discovered somatic mutations in TERT genes which are used to produce Tert and control the Telomeres during cell reproduction. In addition they authors argue that these mutations result from UV radiation.

In this paper we review the basics of Telomeres and then consider the results of the work regarding its influence in melanoma.

It is important to note, as we do herein, that there have been ongoing discoveries of somatic gene mutations found in melanoma over the past decade. There are ligands, receptors, extracellular matrix elements, cofactors from benign cells, internal pathway breaks, transcription factors, miRNAs, and the list goes on. Each time the Press, all too often, with the cooperation of the researchers, makes this most recent discovery a sine qua non. However at best each discovery is but one more step in putting a very complex process in context.

As we have previously, we use extensive primary source references to make our points. Thus this paper provides a window to what we currently understand about this specific issue.

The focus on telomeres and cancer has been an area of active interest for almost two decades. As Shay et al (2012) state:

To grow indefinitely, human cancer cells must counteract the progressive loss of telomeric DNA that universally accompanies cell division. To do this, about 85 to 90% of cancers use telomerase, an enzyme that synthesizes the tandem 5′-TTAGGG-3′ hexanucleotide repeats of telomeric DNA by reverse transcription using its own RNA subunit as a template. Because telomerase is not expressed in most normal human cells, telomerase inhibition is considered an almost universal oncology target, and several clinical trials are under way

The above focuses on the critical importance of telomerase. Before continuing it is worth reviewing the telomere. As Shay and Wright state:

Telomeres are tracts of repetitive DNA (TTAGGG/ AATCCC for human telomeres) that protect chromosomes from degradation and loss of essential genes, and allow the cell to distinguish between double-strand breaks and natural chromosome ends. Human telomeres at birth contain 15–20-kilobase pairs of the repetitive sequence TTAGGG followed by a 3′ single-strand overhang on the G-rich strand, which is believed to be inserted within the double-stranded region to give a lariat-like structure called a t-loop.

Telomeres progressively shorten in most human cells with increased age, and telomere length in almost all middle-aged human tissues is approximately half that of the new born length. Telomere-specific proteins (such as protection of telomeres-1 (POT1), telomeric repeat-binding factor-1 (TRF1) and TRF2) bind directly to the single- and double-strand telomere regions to form a complex, providing a cap over the ends of the chromosomes that protects chromosome termini from degradation, recombination and end-joining reactions.

The authors further state that telomeres are somewhat maintained in humans via the use of telomerase as follows:

Telomere length is maintained by a balance between processes that lengthen telomeres, such as the activity of the cellular ribonucleoprotein enzyme complex telomerase, and processes that shorten telomeres, such as incomplete synthesis of the lagging DNA strand and end processing events. Telomerase stabilizes telomere length by adding TTAGGG repeats onto the telomeric ends of the chromosomes, thereby compensating for the continued erosion of telomeres that occurs in its absence.

Human telomerase contains two essential components, a telomerase reverse transcriptase catalytic subunit (hTERT) and a functional telomerase RNA *(hTR, also known as TERC…*

Other earlier authors such as Campisi et al state:

Telomeres are the repetitive DNA sequences and specialized proteins that form the distinctive structure that caps the ends of linear chromosomes. Telomeres allow cells to distinguish the

chromosome ends from double strand DNA breaks. The telomeric structure prevents the degradation or fusion of chromosome ends, and thus is essential for maintaining the integrity and stability of eukaryotic genomes. In addition and perhaps less widely appreciated, telomeres may also indirectly influence gene expression.

The length, structure and organization of telomeres are regulated by a host of telomere-associated proteins, and can be influenced by basic cellular processes such as cell proliferation, differentiation, and DNA damage. In mammalian cells, telomere length and/or telomere structure have been linked to both cancer and aging. Here, we briefly review what is known about mammalian telomeres and the proteins that associate with them, and discuss the cellular and organismal consequences of telomere dysfunction and the evidence that cells with dysfunctional telomeres can contribute to cancer and aging phenotypes.

Thus the Telomere and its limiting characteristics is a natural target for investigation in cancer research.

4.8.1 Current Contribution

We now move to the two papers which were the focus of this paper. We first examine how they were handled in the press and we will then proceed to examining them first hand.

Let us first start with what was as reported in the Harvard Gazette where we have[61]:

Two mutations that collectively occur in 71 percent of malignant melanoma tumors have been discovered in what scientists call the "dark matter" of the cancer genome, where cancer-related mutations haven't been previously found….

This non-coding DNA, much of which was previously dismissed as "junk," accounts for 99 percent of a cell's genome. A large number of oncogenic mutations in cancer have been identified in the past several decades, but all have been found within the actual genetic blueprints for proteins….

"In addition, this represents the discovery of two of the most prevalent melanoma gene mutations. Considered as a whole, these two TERT promoter mutations are even more common than BRAF mutations in melanoma. Altogether, this discovery could cause us to think more creatively about the possible benefits of targeting TERT in cancer treatment or prevention," Garraway said.

The mutations affect a promoter region — a stretch of DNA code that regulates the expression of a gene — adjacent to the TERT gene. TERT contains the recipe for making telomerase reverse transcriptase, an enzyme that can make cells virtually immortal, and is often found overexpressed in cancer cells. A promoter region of DNA controls the rate of a gene's transcription — the copying of its DNA recipe into a message used by the cell to manufacture a protein….

The researchers said the same mutations are present in cell lines from some other malignancies, and that preliminary evidence showed they might be unusually common in bladder and liver cancers. They also noted that the discovery of these important mutations in DNA previously not linked to cancer-causing alterations highlights the value of whole-genome searches of tumor DNA.

This report details one of the two papers. TERT is the gene which allows for potential immortality of the Telomeres. It repairs the ends as they break and if there is enough of it around then the cell can become immortal. Now the essence of this discovery is that a promoter region adjacent to TERT has been mutated so that TERT is turned on all the time, almost. Thus there is an overabundance of TERT and in turn Telomeres never really shorten, and the cell lives each time it goes through mitosis.

Another report on Science 2.0 states[62]:

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[61] http://news.harvard.edu/gazette/story/2013/01/mutations-drive-malignant-melanoma/

They analyzed the genomes of family members and found an identical mutation in the gene for telomerase, an enzyme often called 'immortality enzyme', in all persons studied. Telomerase protects the ends of chromosomes from being lost in the process of cell division and, thus, prevents that the cell ages and dies. The inherited gene mutation leads to the formation of a binding site for protein factors in the controlling region of the telomerase gene, causing it to become overactive. As a result, mutated cells overproduce telomerase and hence become virtually immortal.

This finding prompted the scientists to also look for mutated telomerase genes in non-inherited (sporadic) melanoma, which is much more common than the familial variant. In most of the tissue samples of melanomas of all stages they found alterations in the telomerase gene switch, which the researchers clearly identified as typical consequences of sun exposure. Even though these mutations were not identical to those found in the melanoma family, they had the same effect: overactive telomerase…

This is also confirmed by the surprising incidence of this alteration: The telomerase gene is the most frequently mutated gene in melanoma. "This is something we hadn't expected, because malignant melanoma has been genetically analyzed thoroughly. But this mutation always seems to have been overlooked," says Kumar.

It should be noted in the above the reference to sun exposure. The argument is that the telomerase change is a direct consequence of the UV exposure. We will focus on that observation later. The "overlooked" nature of this gene and its product is also of issue in that many researchers have examined telomerase extensively so frankly it is not truly new, even as a target for control.

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62 http://www.science20.com/news_articles/familial_gene_mutation_immortalizes_malignant_melanoma-101871

4.8.2 TERT

Before continuing it is worth a quick summary of TERT, the telomerase that maintains the telomere. TERT is located at 5p15.33. From NCBI we have[63]:

Telomerase is a ribonucleoprotein polymerase that maintains telomere ends by addition of the telomere repeat TTAGGG. The enzyme consists of a protein component with reverse transcriptase activity, encoded by this gene, and an RNA component which serves as a template for the telomere repeat. Telomerase expression plays a role in cellular senescence, as it is normally repressed in postnatal somatic cells resulting in progressive shortening of telomeres. Deregulation of telomerase expression in somatic cells may be involved in oncogenesis.

Studies in mouse suggest that telomerase also participates in chromosomal repair, since de novo synthesis of telomere repeats may occur at double-stranded breaks. Alternatively spliced variants encoding different isoforms of telomerase reverse transcriptase have been identified; the full-length sequence of some variants has not been determined. Alternative splicing at this locus is thought to be one mechanism of regulation of telomerase activity.

The observation can be made that if we do not have adequate TERT then the Telomere ends decay and ultimately the cell line dies off. This is the typical case. Therefore take a malignant melanoma cell. If it has in its pathways and receptors been activated to mitotic duplication then if the TERT is inadequate then the Telomere ends get cut shorter each time it goes through mitosis and at some point it just stops.

For example, and this is just for exemplar purposes only, we have a malignant melanocyte, then it goes through mitosis say 10,000 times but each time it would lose a piece of the Telomere until they are all gone, then th cell cannot go again. But if there is an overabundance of TERT, then the TERT resupplies what may be lost and this cell has no way of stopping, at least due to this factor.

[63] http://www.ncbi.nlm.nih.gov/gene/7015

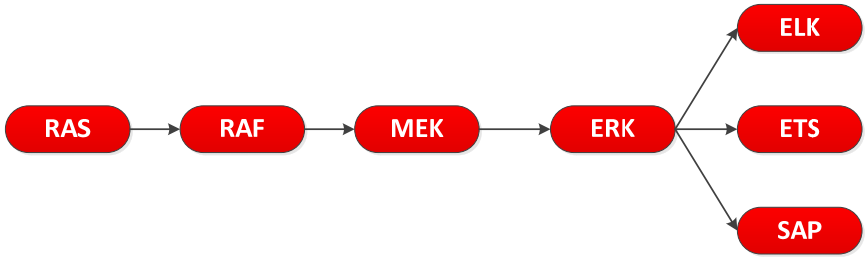
4.8.3 ETS

The ETS family of genes is positive or negative regulators of gene expression. They can up or down regulate expression. They are named for the initial gene discovered, the E26 Transforming Sequence, where E26 was the oncogene v-ets characterized in 1986 of an avian transforming virus called E26. It is also called the erythroblast transforming specific family, as discussed by Zong et al.

The ETS family is a large family of over 20 such genes, and we will focus on ERG specifically. The Table below is from Watson et al.

	Subgroup	Name	Unigene Name	Alternative Names	Locus	Size
1	ETS	ETS1	ETS1		11q23.3	441
2		ETS2	ETS2		21q22.3	469
3	ERG	ERG2	ERG		21q22.3	462
4		FLI1	FLI1	ERGB	11q24.1-q24.3	452
5		FEV	FEV		2q36	238
6	PEA3	PEA3	ETV4	E1AF, PEAS3	17q21	462
7		ERM	ETV5		3q28	510
8		ER81	ETV1		7p21.3	458
9	ETV	ER71	ETV2	ETSRP71	19q13.12	370
10	TCF	ELK1	ELK1		Xp11.2	428
11		SAP1	ELK4		1q32	431
12		NET	ELK3	SAP2, ERP	12q23	407
13	GABP	GABP α	GABPA	E4TF1	21q21.3	454
14	ELF1	ELF1	ELF1		13q13	619
15		NERF	ELF2	NERF1, NERF2, EU32	4q28	581
16		MEF	ELF4	ELFR	Xq26	663
17	SPI1	SPI1	SPI1	PU.1, SFPI1, SPI-A	11p11.2	264
18		SPIB	SPIB		19q13.3-q13.4	262
19		SPIC	SPIC		12q23.2	248
20	TEL	TEL	ETV6		12p13	452
21		TEL2	ETV7	TEL-B	6p21	264
22	ERF	ERF	ERF		19q13	548
23		PE-1	ETV3	METS	1q21-q23	250
24	PDEF	PDEF	SPDEF		6p21.3	335
25	ESE	ESE1	ELF3	ESX, JEN, ERT, EPR1	1q32.2	371
26		ESE2	ELF5		11p13-p12	255
27		ESE3	EHF	ESEJ	11p12	300

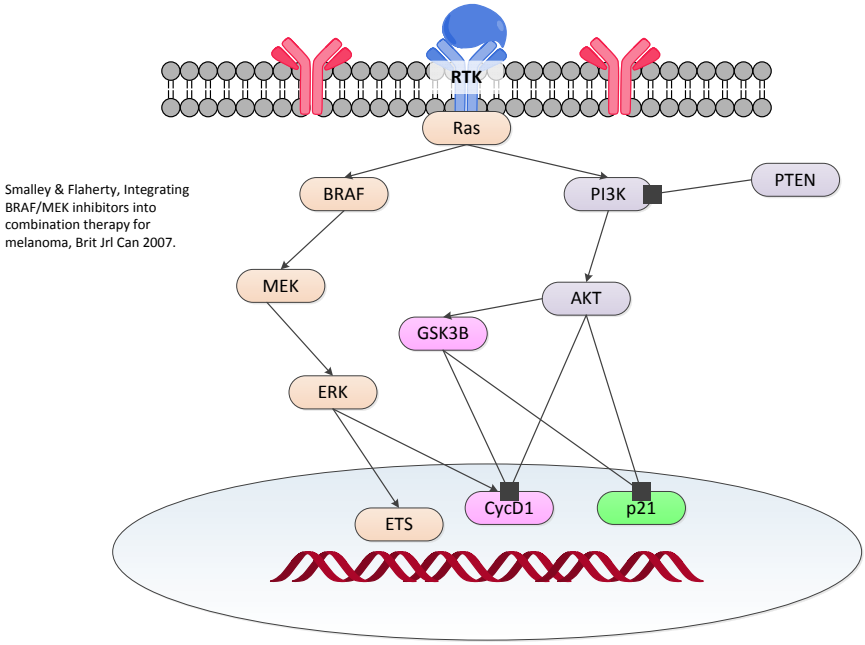
The ERG gene was first presented in the paper by Shyam and Reddy et al in 1987. There the authors identified it and set it in the ETS family. From Weinberg, we see that the ETS are transcription factors driven by the RAS/RAF pathway along with other such factors.



ETS also plays a significant role in the process. We briefly review that as well. ETS is located at 11q23.3. From NCBI we have[64]:

This gene encodes a member of the ETS family of transcription factors, which are defined by the presence of a conserved ETS DNA-binding domain that recognizes the core consensus DNA sequence GGAA/T in target genes. These proteins function either as transcriptional activators or repressors of numerous genes, and are involved in stem cell development, cell senescence and death, and tumorigenesis. Alternatively spliced transcript variants encoding different isoforms have been described for this gene

From Smalley and Flaherty we have the following pathway for ETS:



The mutations we discuss here are somewhat new and they are present in a relatively large number of samples, at least percentage wise. We know that ETS has transcription control and we

64 http://www.ncbi.nlm.nih.gov/gene/2113

can see from above the relationship to BRAF as well. Thus there are many points of loss of control in a melanoma cell. Specifically, as Chudnovsky et al note[65]:

Multiple genetic alterations occur in melanoma, a lethal skin malignancy of increasing incidence. These include mutations that activate Ras and two of its effector cascades, Raf and phosphoinositide 3-kinase (PI3K). Induction of Ras and Raf can be caused by active N-Ras and B-Raf mutants as well as by gene amplification. Activation of PI3K pathway components occurs by PTEN loss and by AKT3 amplification.

Melanomas also commonly show impairment of the p16 (INK4A)-CDK4-Rb and ARF-HDM2-p53 tumor suppressor pathways. CDKN2A mutations can produce p16(INK4A) and ARF protein loss. Rb bypass can also occur through activating CDK4 mutations as well as by CDK4 amplification. In addition to ARF deletion, p53 pathway disruption can result from dominant negative TP53 mutations. TERT amplification also occurs in melanoma.

The extent to which these mutations can induce human melanocytic neoplasia is unknown. Here we characterize pathways sufficient to generate human melanocytic neoplasia and show that genetically altered human tissue facilitates functional analysis of mutations observed in human tumors.

[65] http://www.ncbi.nlm.nih.gov/pubmed/15951821?dopt=Abstract

4.8.4 The Results

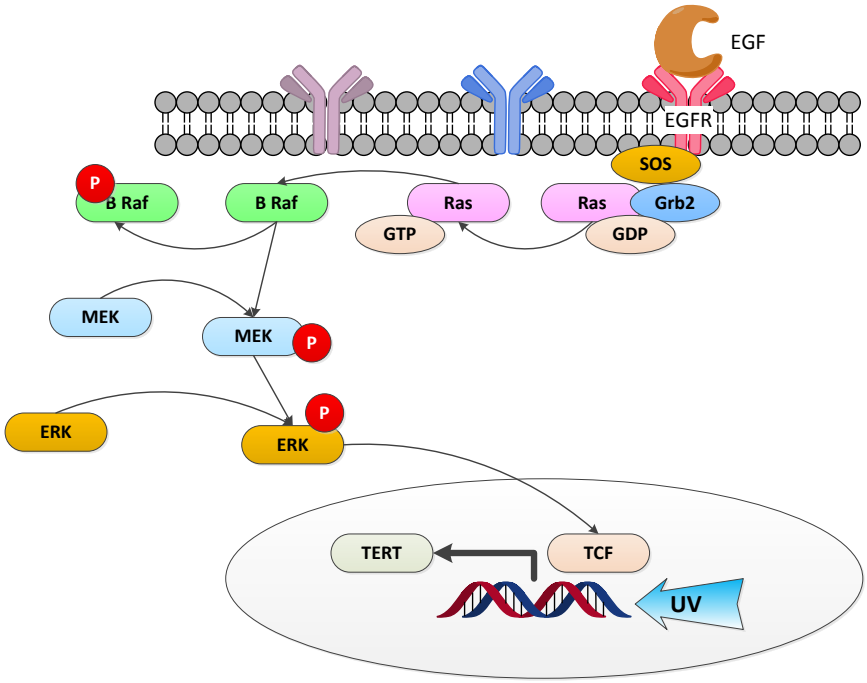
Now we will examine the two recent papers as published in Sciencexpress in early 2013. We start with the paper by Horn et al and then proceed to the second paper.

As Horn et al state:

Cutaneous melanoma occurs in both familial and sporadic forms. We investigated a melanoma-prone family through linkage analysis and high-throughput sequencing and identified a disease-segregating germ line mutation in the promoter of the telomerase reverse transcriptase (TERT) gene, which encodes the catalytic subunit of telomerase. The mutation creates a new binding motif for Ets/TCF transcription factors near the transcription start and in reporter gene assays, caused up to 2-fold increase in transcription.

We then screened the TERT promoter in sporadic melanoma and observed recurrent UV signature somatic mutations in 125/168 (74%) of human cell lines derived from metastatic melanomas, corresponding metastatic tumor tissues (45/53, 85%) and in 25/77 (33%) primary melanomas. The majority of those mutations occurred at two positions in the TERT promoter and also generated binding motifs for ETS/TCF transcription factors.

Horn et al conjecture the following pathway:



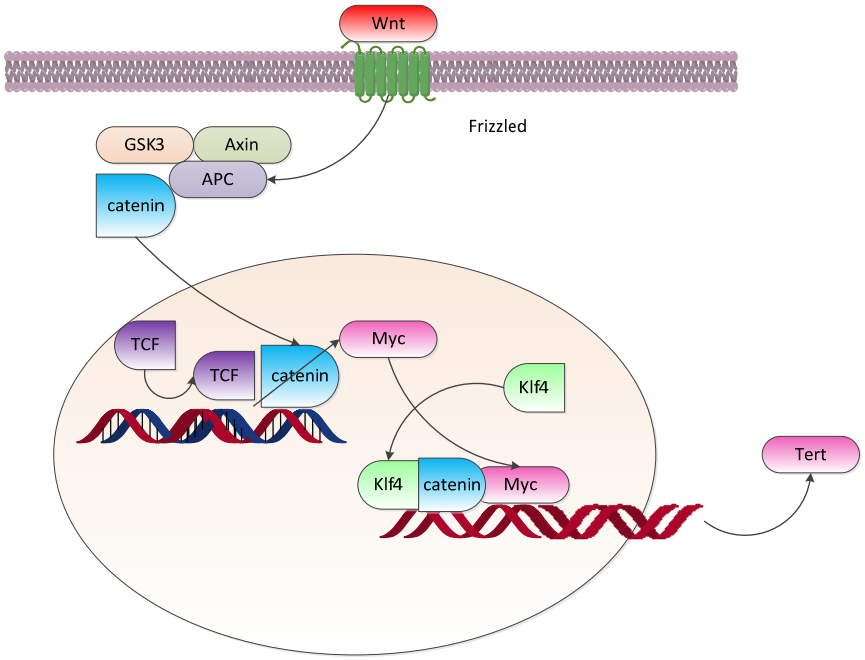
As Huang et al state:

Systematic sequencing of human cancer genomes has identified many recurrent mutations in the protein coding regions of genes but rarely in gene regulatory regions. Here we describe two

independent mutations within the core promoter of TERT, the gene coding for the catalytic subunit of telomerase, which collectively occur in 50 of 70 (71%) of melanomas examined.

These mutations generate de novo consensus binding motifs for ETS transcription factors, and in reporter assays the mutations increased transcriptional activity from the TERT promoter by 2 to 4-fold. Examination of 150 cancer cell lines derived from diverse tumor types revealed the same mutations in 24 cases (16%), with preliminary evidence of elevated frequency in bladder and hepatocellular cancer cells. Thus, somatic mutations in regulatory regions of the genome may represent an important tumorigenic mechanism.

We have discussed before the Wnt pathway connection to TERT as well. As shown below we have discussed this option as well.



This has been discussed by Hoffmeyer as well as by Greider. As Greider states:

Recent studies have proposed that the Wnt pathway is linked to TERT in a quite different way. Constitutive overexpression of TERT in mice activates the Wnt pathway, suggesting that TERT may also function as a transcription factor. Although one study did not observe Wnt pathway activation in response to TERT overexpression, other studies have raised questions about the physiological relevance of the constitutive overexpression of TERT.

Deletion of TERT in mice does not affect expression of target genes in the Wnt pathway, nor give rise to the cellular phenotypes that loss of Wnt signaling induces, indicating that TERT regulation of Wnt signaling may be limited to situations where TERT is overexpressed.

It is reasonable to propose that Wnt regulates TERT given that Wnt signaling plays an essential role in stem cell self-renewal and that TERT is needed for the long-term growth of stem cells. TERT regulation seems to require not one, but two master transcriptional regulators to assure

that there is neither too much, which may allow the growth of cancer cells, nor too little, which might lead to stem cell failure. The finding by Hoffmeyer et al. that both β-catenin and Klf4 are required to activate TERT expression puts the horse (Wnt) before the cart (TERT) and provides a foundation for linking telomerase levels and self-renewal.

Thus TERT regulation is truly a complex process. We have examined the impact of Wnt on melanoma previously. This recent work is on mutations on TERT genes yet we also must consider the influence of Wnt as well.

4.8.5 Observations

This discovery leads to several observations of note:

1. One could have imagined something of this happening with Telomeres. It would almost be necessary to allow ongoing uncontrolled mitotic activity. Thus, despite the fact that there is no surprise here we do have a specific target, namely the activator of TERT.

2. Melanoma, as most other cancers, has a multiplicity of changes to genes. There are ligands, receptors, pathway elements, transcription factors, and the telomere issues as well. It is clear that no single factor is the dominant one as of yet. BRAF as a target works for a while and then there is a work around. Thus cancer is an evolving process, and one which may be highly adaptive.

3. A Conjecture: As we have learned more and more as to aberrant genes and their products, as well as miRNAs, and their effects, one could envision several uses of malignancy profiling. We consider that in two steps.

Step 1: Profiling a Specific Patient at Various Locations. As shown below we consider a specific patient and then profile gene expression as a function of distance from the site of initiation, if such was possible. Then we can see how various aberrant genes are being expressed over the distances measure from the source. One would suspect that distance must be measured in some normalized manner but we leave that as an exercise for the student at this time. This gives us a profile for a specific patient, perhaps one for developing therapeutics.

Specific Patient at Different Locations From Source

Gene	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
G1	7	6	6	5	5	5	5	4	4	4	3	3	3	2	2	2	1	1	1
G2	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
G3	6	6	5	5	4	3	2	1	1	1	1	1	1	1	1	1	1	1	1
G4	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
G5	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
G6	6	6	6	5	5	4	4	3	3	2	2	1	1	1	1	1	1	1	1
G7	7	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
G8	7	4	4	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1
G9	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
G10	8	7	6	6	6	5	5	5	5	5	4	4	3	3	3	3	3	2	1
G11	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
G12	5	5	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
G13	6	5	4	3	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1
G14	7	7	6	6	6	6	5	5	4	4	3	3	3	2	2	2	2	2	2
G15	2	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Step 2: The Same Location but across a Large Pool of Patients: Again we look now at the same distance from the source, perhaps at the same time, again an exercise for the student, and we get profiles of the expression of aberrant genes. This allows us to understand the between patient differences.

		Different Patients at Specific Location from Source																		
Gene		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
	G1	4	3	4	4	3	2	4	5	5	6	6	4	3	2	3	4	4	2	1
	G2	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
	G3	1	3	4	5	2	2	4	3	2	3	4	5	3	2	5	2	3	4	5
	G4	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
	G5	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
	G6	3	4	5	5	6	2	3	5	5	5	5	7	6	8	8	6	8	8	8
	G7	2	5	4	2	3	4	5	2	2	4	3	2	3	4	5	3	2	5	2
	G8	1	3	2	2	1	1	1	1	1	2	3	2	4	3	3	2	1	3	2
	G9	3	5	7	7	7	7	3	5	8	8	8	5	6	7	7	7	7	4	6
	G10	5	2	3	4	5	5	5	5	6	6	6	6	6	6	4	4	5	3	5
	G11	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
	G12	4	5	6	6	5	7	7	3	4	5	5	6	6	7	6	7	6	5	5
	G13	1	5	3	3	4	6	5	5	5	4	3	2	2	1	5	4	4	3	4
	G14	5	7	8	8	6	7	7	8	8	8	7	7	9	8	8	9	7	8	8
	G15	1	2	2	2	1	1	2	2	2	2	1	1	1	2	2	2	3	2	3

3. Is Seventy Enough? The study did an analysis on 70 lesions. Perhaps that is not enough. Furthermore based upon our previous comments perhaps a correlative study is demanded as well, by patient and by distance.

4. One of the problems I see is the continually hyping of the results as if this is finally the right answer. Anyone even slightly familiar with the field will understand that each input is vital but assembling them in a cohesive whole is essential. The systems approach is the sine qua non, but that cannot be done without the continual bench work required to understand the details.

For example in an article in the Boston Globe the reporter states[66]:

Now scientists working independently in Boston and Germany have made a surprising discovery: a set of genetic mutations found in most melanomas, the deadliest skin cancer. The presence of these mutations in the vast majority of tumors studied suggests that the researchers may have stumbled upon a fundamental mechanism involved in a hallmark trait of cancer cells—their ability to live forever—that could one day be targeted by drugs.

Outside researchers said the work, published online Thursday in the journal Science Express, is exciting because the conclusion is the opposite of what many exhaustive studies of cancers have shown.

66 http://www.boston.com/news/science/blogs/science-in-mind/2013/01/24/boston-researchers-discover-mutations-that-underlie-melanoma-junk-dna/mNIYVavGfVsvstVj5eNfzO/blog.html

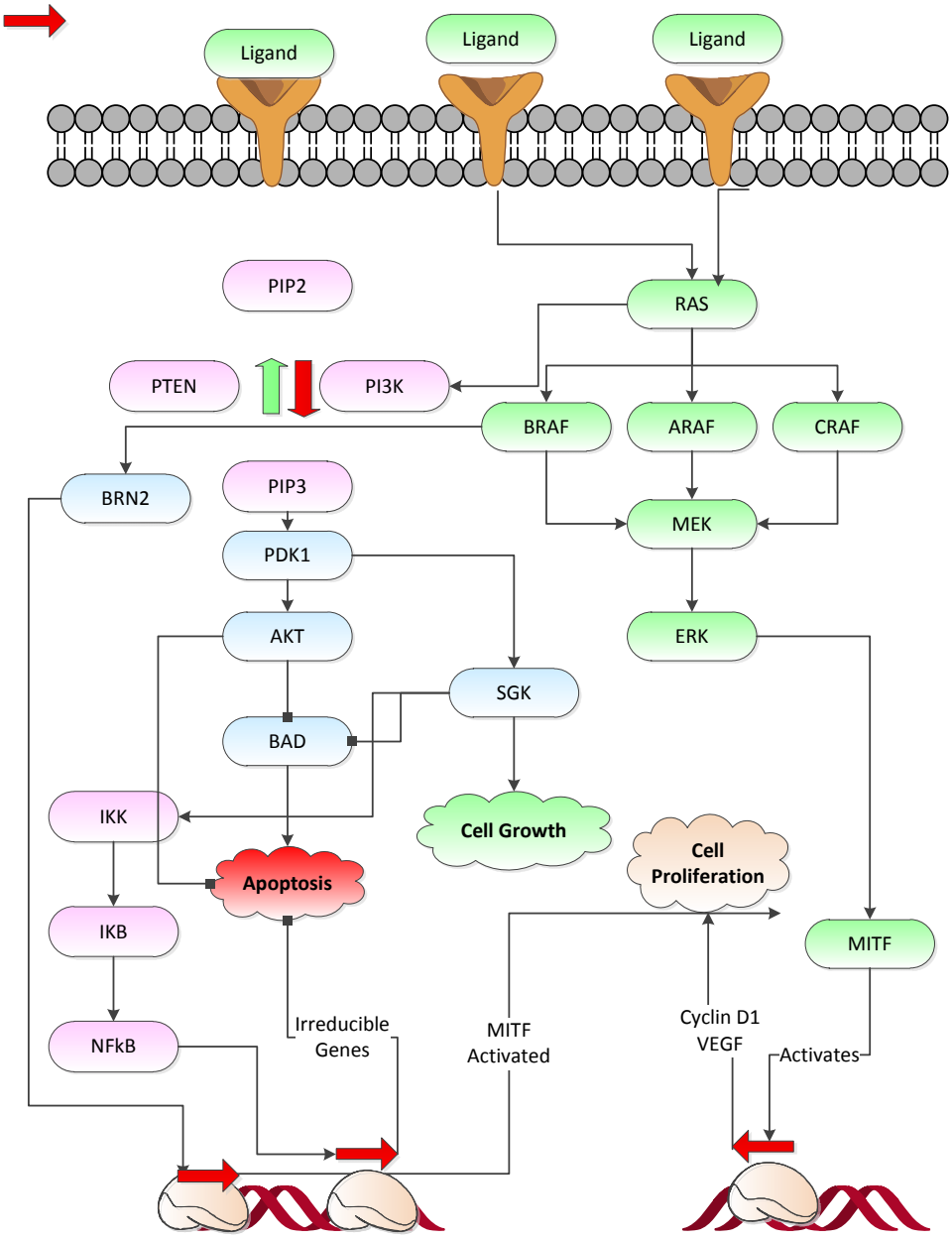
In reality as we have discussed, it was imperative that the Telomeres be preserved in metastasis. Millions of rapid mitotic changes in a stem cell must survive and that means keeping Telomeres and that means lots of TERT. Somehow the conclusion was logical, consistent and not at all unexpected especially given what else has been found in the past decade.

The article continues:

Both teams zeroed in on mutations in a part of the genome called a promoter, which acts like a volume knob on a stereo to control gene activity. The gene that the promoter controlled happened to be one that has long been of interest in cancer because it creates part of an enzyme called telomerase, which enables cancer cells to continue to divide indefinitely as one of its key jobs. Still, it wasn't easy for the researchers to convince themselves that what they found, underlying more than two-thirds of melanoma cases, was real.

One would expect this and if one looks at say the miRNA discoveries, they all add up to what controls the ultimate expression of mitotic survival.

5. Theraputics: Can we expect therapeutics from this understanding? Good question. Kinase inhibitors are now well understood, one could in theory build an inhibitor here as well. Is this the target, another target, necessary, helpful, we can only guess. Yet the above Conjecture may allow for the development of a therapeutic profiling plan for melanoma and other malignancies.



Simply there are three end states:

1. Cell Proliferation or Cell Cycle Mitosis

2. Cell Growth or the expansion and operations of a single cell outside of mitosis.

3. Apoptosis or cell death.

Now in the simplified model above we have several feedback loops, many driven by external ligands.

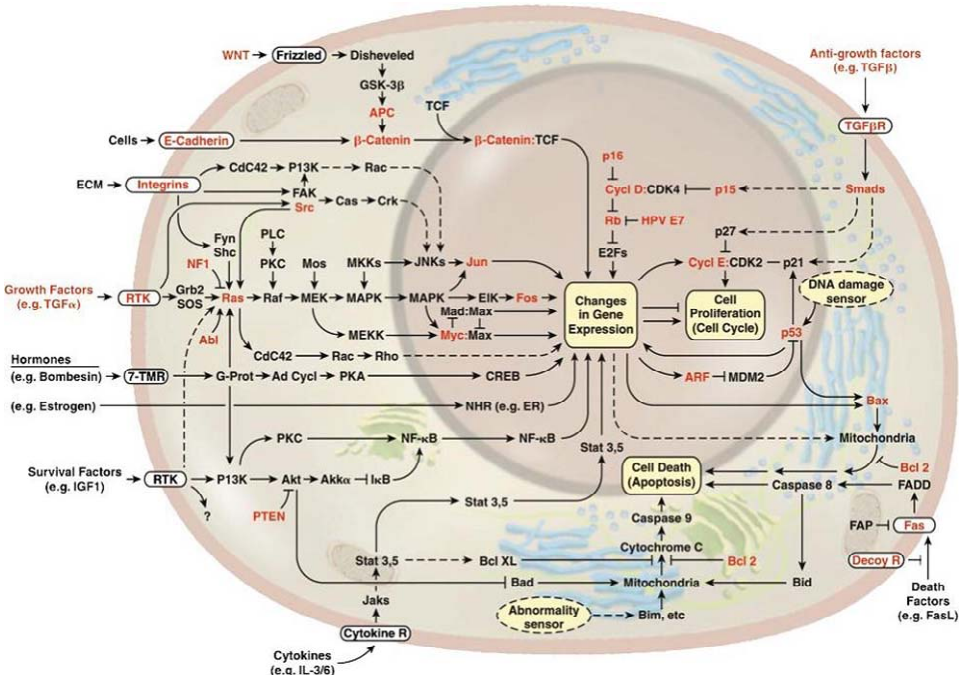
In this section we briefly review the issue of cellular growth. What makes cells reproduce. Skin cells are reproducing all the time. Mostly the keratinocytes and getting sloughed off at the surface or rebuilding after a wound. The melanocytes frequently do not reproduce. They are neural crest derives and often just remain in the G0 state. They produce such products as melanosomes, and other proteins required for homeostasis. There are times when they may reproduce to a cluster state, such as found in lentigenes. This is a common response to excessive sun exposure. Namely we may see heavily pigmented areas of clustered melanocytes. Then we may have a nevus, the raised collection of melanocytes. In both cases the melanocytes tend to stay attached to the cluster, thus having functional E cadherin molecules.

In melanoma, however, we see loss of spatial stability and the cells proliferate, grow and propagate. Furthermore there is little if any cell death, apoptosis.

5 INTRACELLULAR PATHWAYS

In this chapter we examine the many intracellular pathways we find in all cells and then we focus on those pathway elements which are melanoma relevant. It is essential to note that what we do here is work on developing the pathways which are found within a cell, and we examine each of them in some detail so that when we examine a melanoma cell we have a model of control against which to judge. Our approach is dramatically different than many who just take melanoma cells and identify aberrant genes en masse. Namely one can identify many genes which are found in melanoma yet their presence without having a control pathway model may very well be meaningless.

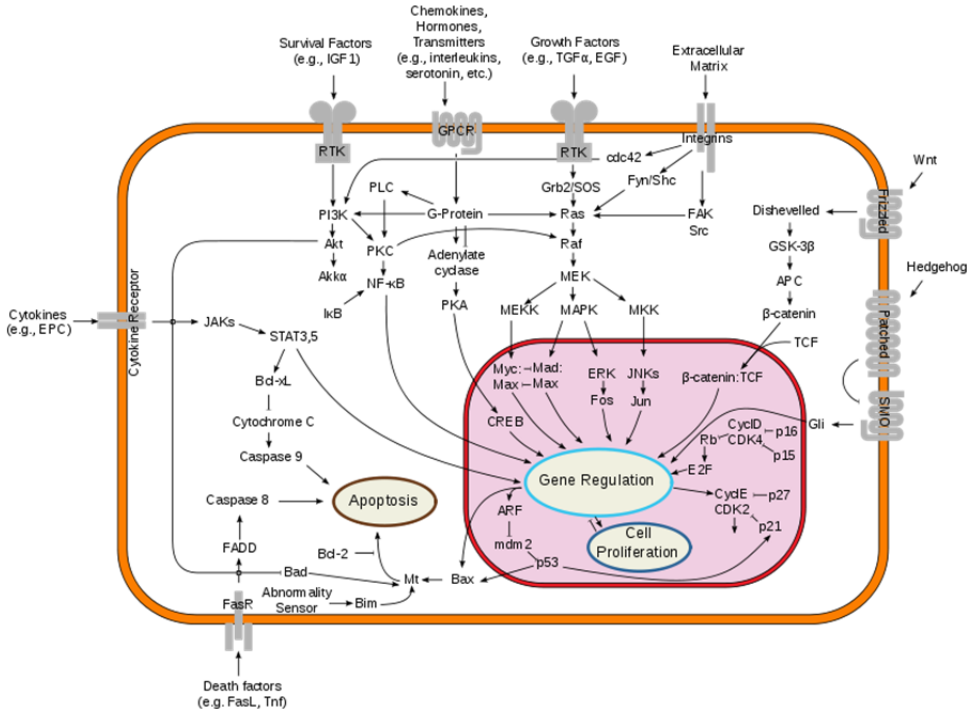
The following is a generic picture of known dominant pathways and receptors. We shall examine them in some detail. This model shows proteins, gene products, and then details their specific interactions. It should be noted that it does not show temporal characteristics or concentration effects which we have discussed earlier. This chapter focuses on these pathways as regards to homeostatic and aberrant behavior.



We will examine each of the major pathway elements for which we have evidence that melanoma is related thereto. There may very well be pathways and their elements for which melanoma has no relationship, and further there may be pathways and elements for which we currently have no evidence. In addition there may very well be pathways for which we have no current knowledge as well. Thus the presentation contained herein has a twofold characteristic; first it is temporally bound by what is known when this was prepared, second, and more

importantly, it establishes a paradigm on how to consider pathways and their elements to build and test a more robust model.

A somewhat more simplified model is shown below:

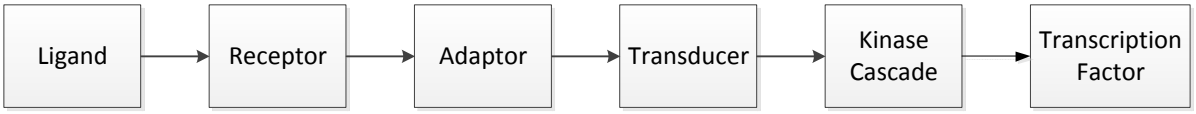


Note that we can envision several categories of proteins, gene products, namely: (i) external proteins called ligands, (ii) surface proteins called receptors, (iii) cytoplasm proteins called pathway proteins, and (iv) nucleus based proteins often being transcription factors. This is an input/output system. Namely, we have proteins in and proteins out, with proteins flowing internally in a complex control fabric. The goal of any cell is survival and proliferation. In cancer cells the cell manages to spread itself out with a total disregard for the rest of the cells in the body. We frequently use the metaphor of a separate organism, like a slime mold, growing upon the normal body elements. Unlike the well-controlled collection of interconnected homeostatic cells, the cancer cells proliferate and move with total disregards to where they may go and what functions the surrounding environment is involved in.

We can envision the overall process by the model below:

Ligand	PDGF	Insulin	Growth Hormone	IL-1β	TGF-β
Receptor	PDGF Receptor	Insulin Receptor	GH Receptor	IL Receptor	TGF Receptor
Adaptor	SHP2/Grb2	IRS 1			
Transducer	SOS/Ras	PI3K	JAK	JAK	Type 1 Receptor
Kinase Cascade	MAPK	Akt			
Transcription Factor	Ternanry complex factors	FOXO	STATs	STATs	SMADs

See p 818 Lewin



Note here we have the generic pathway model. It functions as follows:

1. A ligand is generally a protein which is extra cellular. It finds its way to the cell outer membrane. We shall discuss this extracellular flow in some detail later. It may not be just some random diffusion but may have factors which direct its movement. This thus must become part of the overall modeling of cancer.

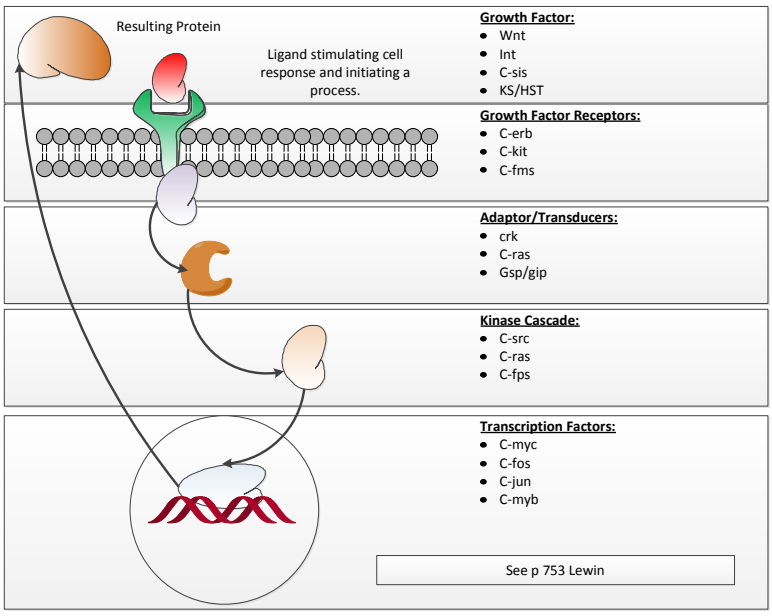
2. A receptor is another trans-membrane protein. It becomes a receiving site for the ligand. which when bound to the receptor becomes an active site in the cell membrane to the outer world. Thus the collection of ligand and receptor is not just a switch but a complex valve process which brings things into the cell and activates things within the cell as well.

3. The adaptor and Transducers are inner cell proteins which manage to connect to the activated receptor and transfer the signals to a set of kinases.

4. Kinases are proteins that move things around. κινο in Greek means to move, actually I move, and kinases are movers and are the proteins which make up the signalling path from the outer cell wall to the nucleus.

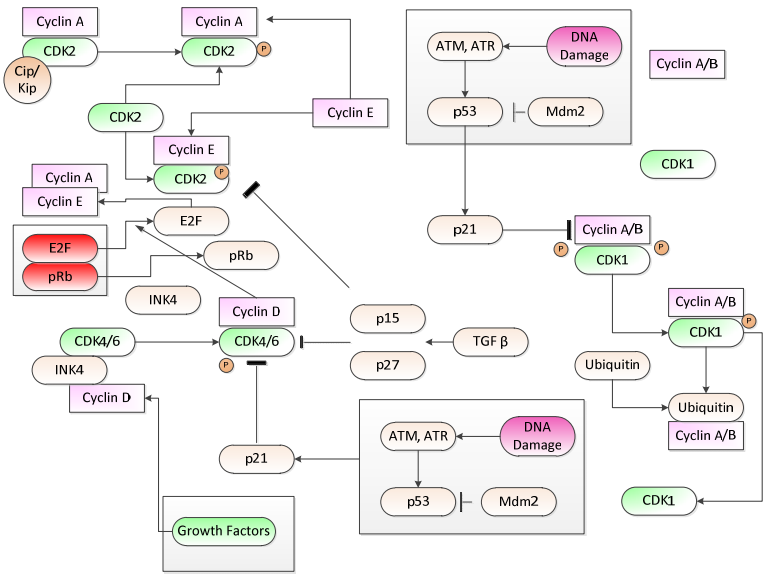
5. Transcription factors are specific proteins which allow for DNA transcription of DNA to RNA as a step to translation, namely RNA to proteins.

We detail this model as follows:



The above graphic is an essential model for what we seek when looking at the cell flow and the pathways. It is critical to understand that this is but one cell.

The following is an example of such a process where we have included the Cyclin molecules which we have discussed as essential to cell reproduction through mitosis. Note we have demonstrated the cyclin development for mitosis and the ubiquitin development for digestion of proteins.



The question is; which genes do we focus on and why? In the chapter by Garraway and Chin in DeVita etal they present a Table of putative melanoma genes (p 347). We repeat this table below:

Gene	*Product Behavior*
INK4A	Tumor suppressors
ARF	Tumor suppressors
NRAS	Oncogenes
BRAF	Oncogenes
PTEN	Tumor suppressor
NEDD9	Metastasis enhancer
MITF	Oncogene
WNT5A	Metastasis enhancer
GOLPH3	Oncogene
ETV1	Oncogene
ERBB4	Oncogene
IGFBP7	Tumor suppressor
GAS1	Metastasis suppressor

There are 13 genes out of somewhat over a hundred that we can observe in the process and out of a total of 23,000 protein encoding genes known to exist. Now as we shall demonstrate herein, as more studies are performed we discover more genes whose alterations are found to occur in melanomas. That frankly is the problem as well. For with the ability to perform GWA, genome wide analysis, we obtain volumes of data on altered genes and researchers then infer causality. This may not be the case.

5.1 THE SINGLE CELL

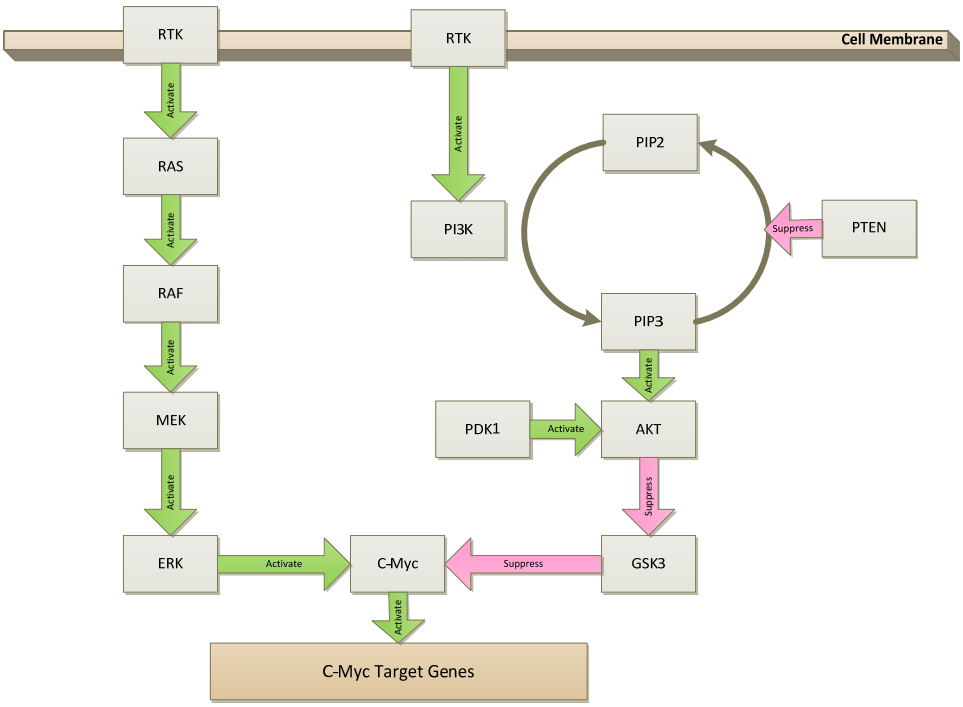
Let us begin with a single cell. We show this below in several simple pathway elements. Here we have three of the possible elements:

Receptors: These shows RTK, receptor tyrosine kinase, (see Markes et al pp 239-249, Kullander and Klein) which are receptors such as EGFR, PDGFR, IR, HGFR, Ret and others.

Pathway Elements: These are the extra nuclear gene products which interact with one another as shown below. This is where the complexity arises. Look at the PTEN function which suppresses one element which ultimately suppresses c-Myc. Since c-Myc is a transcription factor then PTEN can modulate or suppress that factor. The issue here is related to number of such proteins, and then the separate pathways. There is not one PTEN floating in the cytoplasm. There may be dozens, but there may be more PI3K than PTEN and thus PTEN may not work at full potential. Thus the complexity of the control networks. We shall examine this later in the book.

Transcription Elements: Here we show c-Myc. This is one of many such transcription related proteins. It is useful to produce proteins but can be harmful by producing too many.

The following is a simplified Figure depicting two major pathways which we shall return to again and again.



We should then compare this translational model to the cell mitotic model of the previous drawing.

5.2 PUTATIVE GENES

We now will examine some of the genes which are argued to be part of the melanoma process. Unlike the Vogelstein model for colon cancer, where there is a progressive set of mutations required to progress to a carcinoma, in melanoma there is as of yet no clear model. There is however a collection of putative genes whose loss of function result in melanoma. Actually many of the analyses are based on genome wide analysis ("GWA") of various melanoma cells. The problem with a GWA is that we may find genes which have been altered but for which there is as of yet no clear pathway model.

Moreover one must understand that cancer is reflected in loss of localization, loss of mitotic control, loss of apoptosis, and loss of functionality. These functions are complex and have multiple genes in their control paths. Furthermore we do not understand what causes the gene changes. Putatively UV radiation or even low density X-rays, such as found in backscatter, may at the right time be the cause. We then add the issue of a stem cell, and then we may be finding lots of aberrant genes but not on the stem cells. Finally epigenetic factors such as miRNA and methylation may cause pathway blockage even when no gene change is seen. Thus understanding a holistic model is essential but there are still many empty blocks which must be filled in.

Many authors and researchers have documented various gene targets fund in melanomas. These may be causative or they may be a result of the process. As Ugurel at al state:

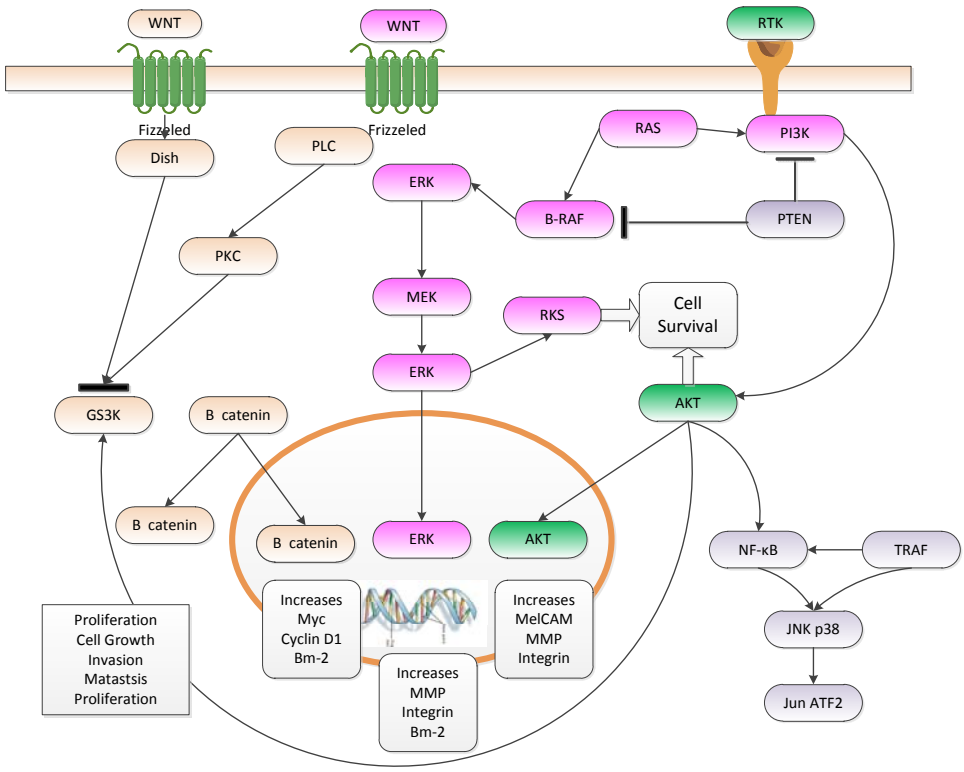
In a recent retrospective study, primary melanomas (for which a long-term clinical follow-up was available) were analyzed using a cDNA expression microarray.[21] The authors described a signature of 174 genes to identify patients at risk of developing distant metastasis. From these genes, 141 were underexpressed and 33 overexpressed in tumors whose host remained free of metastasis for 4 years. Of these 174 genes, 30 had been already studied in melanoma; these genes are involved in cell cycle regulation (CKS2, CDC2, CCNB1, CENPF, and DHFR), mitosis (HCAP-G and STK6), mitotic spindle checkpoint (BUB1), inhibition (BIRC5) or stimulation (GPR105) of apoptosis, DNA replication (TOP2A,RRM2, TYMS, PCNA, MCM4, and MCM6), stress response (GLRX2, DNAJA1, HSPA4, HSPA5, HSPD1, and TXNIP), ubiquitin cycle (SIP), actin and calmodulin binding (CNN3), intracellular signaling (STMN2), negative regulation of the Wnt signaling pathway (CTNNBIP1), inhibition of MITF expression (EMX2), regulation of proteolysis (TNA), testis cancer (CML66), and metastasis suppression (NME1).

The following Table depicts the major genes by category and the ones putatively related to melanoma by author to date:

Element	*Type*	*DeVita et al*	*Hearing and Leong*	*Murphy*	*Bunz*	*Schulz*	*Vidwans et al*	*Fecher et al*	*Ugurel et al*	*Recent*
4EBP1	Transcription									
ABL	Pathway									
AKT	Pathway						X	X		
AMPK	Pathway									
APC	Pathway									
ARF	Pathway	X								
BAD	Pathway									
BMP	Pathway									
CCND1	Transcription									
CDK4	Transcription						X	X		
CREB	Transcription									
Disheveled Dsh	Pathway									
E cadherin	Cell Surface									
EGF	Ligand									
EGFR	Receptor				X					
eIF4E	Transcription									
ERBB4	Receptor	X								
ERK	Pathway									
ETV1	Transcription	X								
FGFR	Receptor									
FOS	Transcription									
Frizzled	Receptor									
GAS1	Pathway	X								
GLI2	Transcription									
GOLPH3	Pathway	X								
GR	Receptor									
GSK-3β	Pathway									
Hedgehog	Ligand									
Her2	Ligand									
HGF	Ligand									
HIF	Transcription									
IGFBP7	Ligand	X								
INK4A	Transcription									
IR	Receptor									
JUN	Transcription									
KIT	Receptor				X					
LEF	Transcription									
LKB1	Pathway									X

Element	*Type*	*DeVita et al*	*Hearing and Leong*	*Murphy*	*Bunz*	*Schulz*	*Vidwans et al*	*Fecher et al*	*Ugurel et al*	*Recent*
MAPK	Ligand									
MEK	Pathway							X		
MITF	Transcription	X					X			
mTOR	Pathway									
MYC	Transcription									
NEDD9	Pathway	X								
NF1	Pathway									
NF-κB	Transcription									
Notched	Receptor									
NRAS	Pathway									
p15	Pathway									
p16 (CDKN2A)	Pathway				X	X	X			
p21	Pathway									
p27	Pathway									
p53	Pathway					X	X			
PAR1	Pathway									
Patched	Receptor									
PDGF	Ligand									
PI3K	Pathway						X	X		
PIP2 PIP3	Pathway									
PREX2	Pathway									X
PTEN	Pathway	X			X	X	X	X		
RAF (BRAF)	Pathway	X			X	X	X	X		
RAS (HRAS)	Pathway	X			X	X		X		
S6K1	Pathway									
SMAD4	Pathway									
Smoothened	Receptor									
SPOP	Pathway									
STRAD	Pathway									
TCF	Transcription									
TCF	Transcription									
TGF	Ligand					X				
TOR	Transcription									
TSC1	Pathway									
TSC2	Pathway									
VEGF	Ligand							X		
Wnt	Ligand	X								
β catenin	Pathway									

The following Figure places many of the above in context.



The concerns are then related to how these various elements interact and what happens if there is a change in a gene changing the expression and in turn its point of control.

We will therefore examine the genes most of interest by general type. We will also look more closely at recent finds and examine them in the context of model building.

Our objectives are as follows:

1. Understand and identify the genes which may be causative by stage.

2. Understand and model the pathways and determine what the elements are.

3. It is critical to understand pathways in a dynamic manner. There have been many attempts to model this but as we will demonstrate later they often have substantial deficiencies. We shall provide certain modifications which may lead to a more productive direction.

4. Understanding the epigenetic factors will also become essential.

We begin with a discussion of several of the key receptors and ligands.

5.3 RECEPTORS

Receptors are protein complexes which reside at the cell wall and can on the one hand attract and bond with ligands or intercellular proteins while on the other hand bond or release, in essence

activate, intracellular pathway proteins. They are in essence switch activating points. We shall demonstrate several examples and they can be embodied in various forms.

The receptors we have focused upon include the following:

- EGFR
- ERBB4
- FGFR
- Frizzled
- GR
- IR
- KIT
- Notched
- Patched
- Smoothened
- MER

We shall focus upon a few to demonstrate how they function. Several have been associated with melanoma; EGFR, ERBB4, KIT.

Receptors are activators, for better or worse. We shall demonstrate how some function. Our objective is to develop an understanding or receptors to the degree where we can include them in dynamic models.

We will first examine a specific receptor and the use of inhibitors.

5.3.1 MER, Melanoma and Inhibitors

The focus on pathways, receptors, ligands and promoters as control elements for cancer has seen a great deal of development in the past decade. One key approach is the development and identification of inhibitors, molecules which can block an over excited pathway. We examine here a specific recent such example as relates to melanoma. It is already well known that BRAF suppression is an effective approach albeit often of limited duration. The development of inhibitors for a selection of evolving pathway aberrations will most likely be the way to turn a deadly disease into a chronic but manageable problem, assuming that one can get permission to use such molecules, a process which not is costly and lengthy.

We use a recent paper by Schlegel et al and use MER as a prototypical example of pathway control via inhibitor blockage.

MER is a tyrosine kinase ("TK") receptor ("TKR")[67]. As Marks et al state there are 85 members in the TK family and 58 of these are receptors. The receptors are divided into various families based upon their structures and one family contains Axl, Sky and MER, also known as the TAM

[67] From NCBI we have (2q14.1): *This gene is a member of the MER/AXL/TYRO3 receptor kinase family and encodes a transmembrane protein with two fibronectin type-III domains, two Ig-like C2-type (immunoglobulin-like) domains, and one tyrosine kinase domain.*

family[68]. This family, as we shall see, has immunoglobulin like regions on the outside of the cell surface and kinase domains on the inner surface. The family also has a dual fibronectin III-like domain on the outside just below the immunoglobulin domains, of which there are two.

Generally the receptors are activated by ligands which in turn result in the phosphorlyation of the kinase region and associated area and then commence the activation of the related pathways. Now these pathways are the ones that result in proliferation and loss of localization and thus result ultimately in metastasis.

We use this example for two reasons: (i) it is a good example to demonstrate the activation of pathways and metastatic growth; (ii) it also is a good example of how inhibitors can function on receptors and thus can inhibit metastatic growth.

As Schlegel et al state:

Receptor tyrosine kinases (RTKs) are frequently ectopically expressed, overexpressed, or hyperactivated in tumor cells and are therefore attractive targets for cancer therapy. C-MER proto-oncogene tyrosine kinase (MERTK), a member of the TAM (TYRO, AXL, MERTK) family of RTKs, has been characterized as a therapeutic target in hematopoietic malignancies and several solid tumors including lung, prostate, and brain

There is a subtle question posed but not answered here. Is it over-expression, and if so by what ligand, or is it an excess production of MER and thus an over-expression. What is the status of the benign cell, and is this the dominant pathway. Clearly by having too active or too many MER receptors, actually any TAM like receptor will do, leads to proliferation. This goal of blocking the receptor so that it does not start the process is a valid approach.

The authors clearly state:

Stimulation of melanoma cells with the MERTK ligand GAS6 resulted in the activation of several downstream signaling pathways including MAPK/ERK, PI3K/AKT, and JAK/STAT. MERTK inhibition via shRNA reduced MERTK-mediated downstream signaling, reduced colony formation by up to 59%, and diminished tumor volume by 60% in a human melanoma murine xenograft model.

Namely we have a ligand, GAS6, which activates the MER pathway. Is that ligand over expressed. On the other hand the molecule shRNA reduced the activation.

They specifically state:

In addition, Sensi et al. found that melanoma cells often secrete GAS6, a ligand of TAM receptors, indicating a mechanism of TAM autocrine signaling in melanoma…. The mechanism of MERTK activation in melanoma cells is not clear, but Sensi et al. have previously described melanoma cell expression and secretion of GAS6, the common ligand for all members of the TAM family of proteins, suggesting a method of autocrine and/or paracrine activation of

[68] TAM, (**T**YRO, **A**XL, **M**ERTK)

MERTK. Since expression of MERTK by melanoma cells increases during progression from primary to metastatic melanoma, it would be interesting to determine whether corresponding increases in GAS6 levels occur in serum from patients with metastatic melanoma, implicating serum GAS6 levels as a potential early marker of melanoma progression, as in other cancers.

Thus possibly inhibiting GAS6 may be profitable as well[69]. However the focus here is receptor inhibition.

5.3.2 MER and Melanoma

Let us consider a recent development in understanding MER and melanoma. We return to the recent paper by Schlegel et al where the author's state:

C-MER proto-oncogene tyrosine kinase (MERTK) is a receptor tyrosine kinase with oncogenic properties that is often overexpressed or activated in various malignancies. Using both protein immunohistochemistry and microarray analyses, we demonstrate that MERTK expression correlates with disease progression.

MERTK expression was highest in metastatic melanomas, followed by primary melanomas, while the lowest expression was observed in nevi. Additionally, over half of melanoma cell lines overexpressed MERTK compared with normal human melanocytes; however, overexpression did not correlate with mutations in BRAF or RAS.

Stimulation of melanoma cells with the MERTK ligand GAS6 resulted in the activation of several downstream signaling pathways including MAPK/ERK, PI3K/AKT, and JAK/STAT. MERTK inhibition via shRNA reduced MERTK-mediated downstream signaling, reduced colony formation by up to 59%, and diminished tumor volume by 60% in a human melanoma murine xenograft model.

Treatment of melanoma cells with UNC1062, a novel MERTK-selective small-molecule tyrosine kinase inhibitor, reduced activation of MERTK-mediated downstream signaling, induced apoptosis in culture, reduced colony formation in soft agar, and inhibited invasion of melanoma cells. This work establishes MERTK as a therapeutic target in melanoma and provides a rationale for the continued development of MERTK-targeted therapies.

Thus, like to work that led to BRAF V600 inhibitors, we see MER TK is another interesting target. The authors also provide an inhibitor molecule as well.

5.3.3 Tyrosine Kinases and MER

Tyrosine Kinases receptors have received a great deal of attention especially in the area of cancer metastasis and in cancer control. They are as Verma et al state:

---

[69] As NCBI states: *This gene product is a gamma-carboxyglutamic acid (Gla)-containing protein thought to be involved in the stimulation of cell proliferation, and may play a role in thrombosis. Alternatively spliced transcript variants encoding different isoforms have been found for this gene*. Located at 13q34. http://www.ncbi.nlm.nih.gov/gene/2621

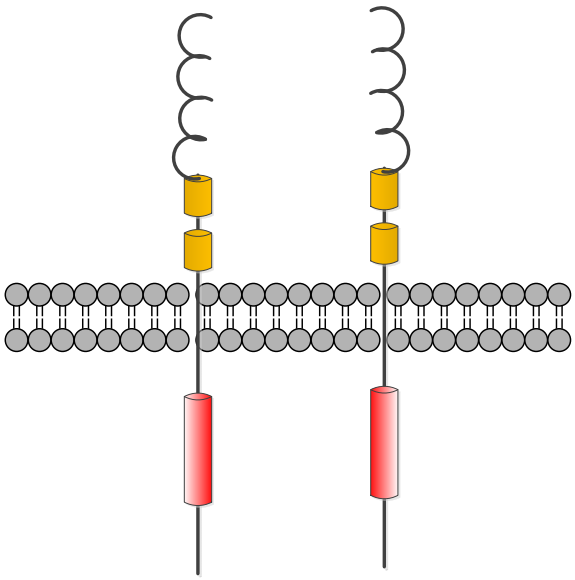
Receptor tyrosine kinases (RTK) are a large family of transmembrane proteins exhibiting great diversity in their extracellular regions, although sharing in common a highly conserved intracellular tyrosine kinase domain. They function as sensors for extracellular ligands, the binding of which triggers receptor dimerization and activation of the receptor's kinase activity. This activation leads to the recruitment, phosphorylation, and activation of multiple downstream signaling proteins, which ultimately change the physiology of the cell. RTKs regulate cellular processes, including survival, growth, differentiation, adhesion, proliferation, and motility. Fifty-eight known RTKs in the human genome are classified into 20 families by amino acid sequence identity within the kinase domain and structural similarities within their extracellular regions.

There are many such tyrosine kinase receptors. One class is the TAM family and as Verma et al state:

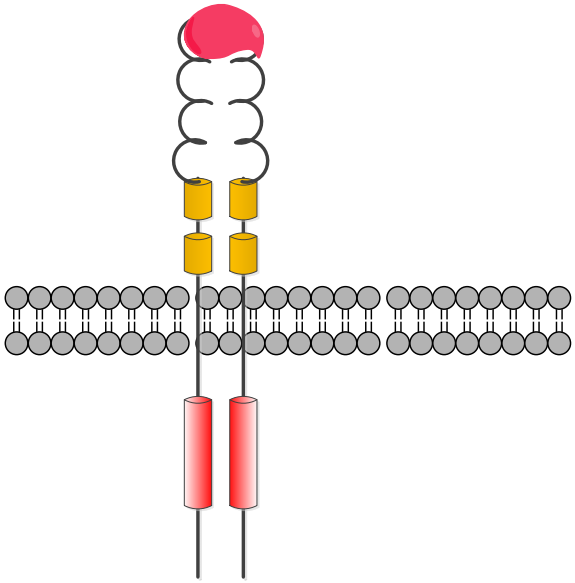
One subfamily is referred to as the TAM family, identified in 1991, comprising Tyro-3 (also called Sky), Axl, and Mer. The TAM receptors are characterized by a combination of 2 immunoglobin-like domains and dual fibronectin type III repeats in the extracellular region and a cytoplasmic kinase domain. The primary ligand for TAM receptors is growth arrest-specific 6 (Gas 6), a fairly large (75 kDa) vitamin K–dependent protein known to activate downstream signaling

We depict a simple structure below containing the elements specified above.

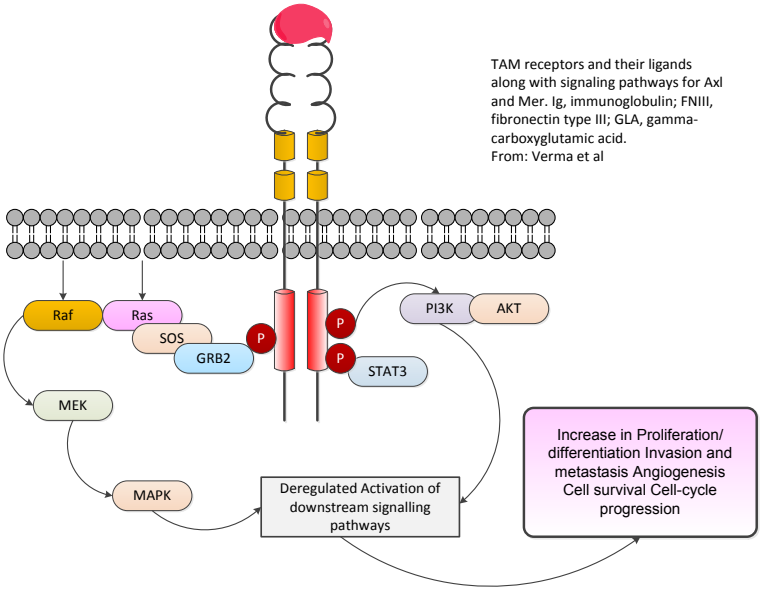
Let us consider a simple development of MER controlled pathways. The Figure below shows two separate and un-activated MERTK molecules with the immunoglobulin terminals on the outside and the kinase areas on the inside.



Now along comes a GAS6 ligand, and it attaches to and connects the MERTK molecules at the immunoglobulin ends and this activates the kinase tails inside the cell.



Once activated the kinase ends commence pathway activation via the phosphorylation process. The pathways are depicted below.



It is the activation of these pathways by the excess GAS6 production or the excess MERTK production or both that results in excess proliferation and metastasis.

As Verma et al relate about the pathway:

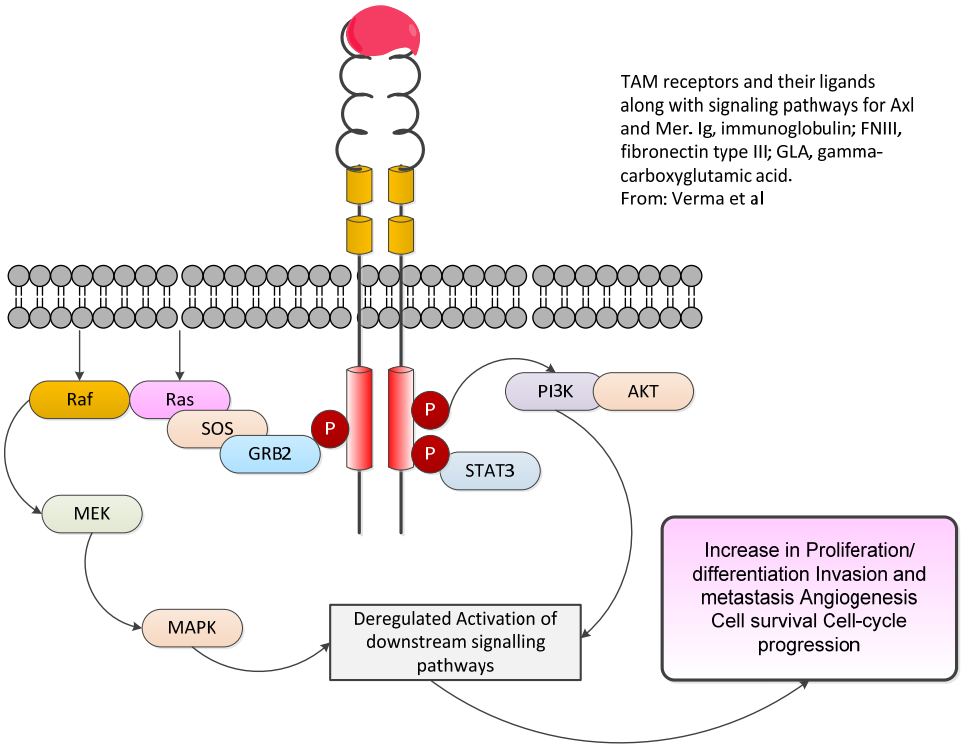
Studies using chimeric Mer receptors expressed in NIH3T3 fibroblasts linked downstream signaling pathways, such as PI3K, phospholipase C-g (PLCg), and ERK, to Mer activation. Gas

6–dependent activation of Mer stimulates phosphorylation of ERK1/2, leading to cellular transformation and increased proliferation and DNA synthesis.

The ultimate downstream targets of the pathway differ according to cell type and tissue microenvironment. In leukemia cells, ligand-dependent activation of EGF receptor (EGFR)–Mer chimeric receptor stimulates phosphorylation of Akt, ERK 1/2, and p38 mitogenactivated protein kinases (MAPK), which results in decreased apoptosis but no change in proliferation (30). Expression of CD8-Mer chimera in pro-B cells results in transcriptional activation of NF-kB via PI3K/Akt.

Additional activation of p38/MAPK and meiosis-specific serine/threonine protein kinase 1 (MEK1) occurs via CD8-Mer, leading to protection from apoptosis. Some atypical signaling pathways involved in cell survival have been studied as a link between Mer and the actin cytoskeleton via growth factor receptor-bound protein 2 (Grb2), Shc, and Vav1. Downregulation of the proapoptotic tumor suppressor WW domain-containing

We depict below how one can inhibit this process. We depict an inhibitor molecule which binds to the sites as before but now does not activate the TK pathways. The inhibitor must be stronger in affinity than the GAS6 which most likely is still in ECM abundance.

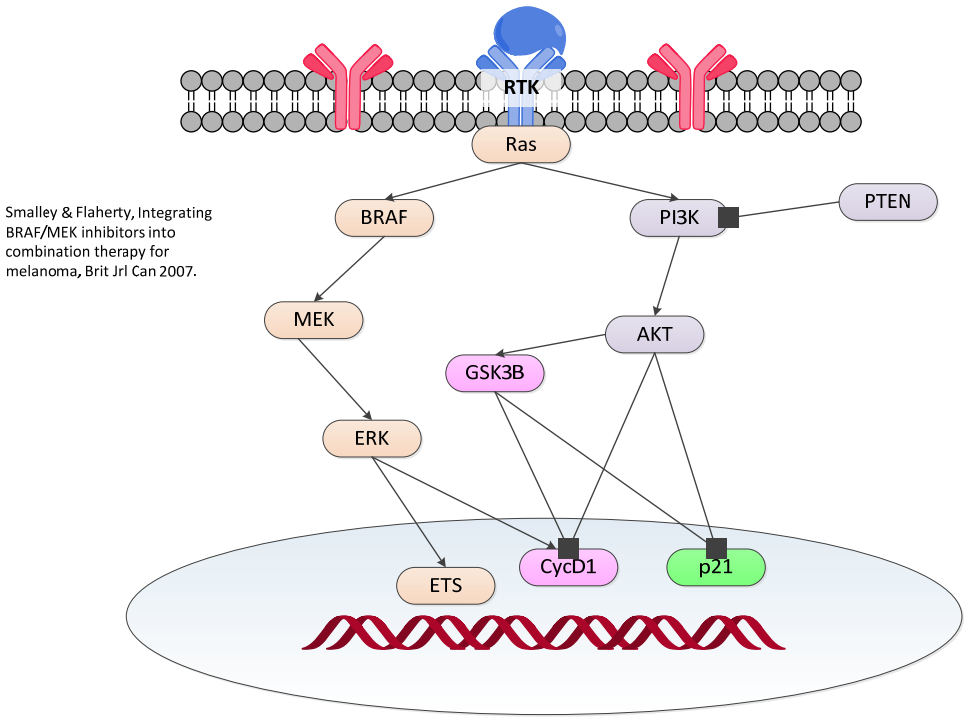


Note above the RAs to RAF (especially BRAF) to MEK to MAPK pathways flow. We have examined this in details elsewhere[70]. The implication is that by targeting the TK Receptor, one targets all elements of the pathway. It should be noted however that the separate pathway

[70] See McGarty, Melanoma Genomics, DRAFT, 2013.

elements may be activated and over expressed via other factors such as epigenetic ones. Thus the suggestions of Schlegel et al are of great merit but should be balanced by understanding the epigenetic issues as well.

An example of a pathway and its control with BRAF functionality is depicted below:



This simple explanation is also a paradigm for many other such pathway activations and especially for those of the tyrosine kinase verity.

5.3.4 MER and miRNA

There are other dimensions of interest here as well. In cancers there unfortunately is not just a single point of failure. There often are multiple. We show here just another example where MER and miRNA play an interesting role. This is an essential point to make because all too often the initial observers may all too often jump at a simple solution leaving behind a complexity of other factors which take control.

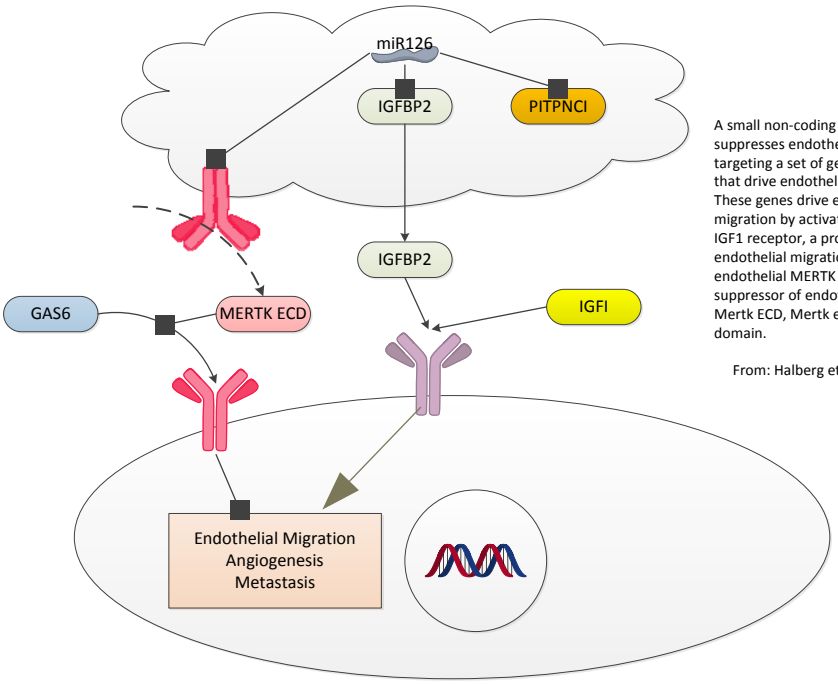
Let us consider a miRNA control using MER. As Halberg et al state:

Tumours require the establishment of vasculature for their increasing nutrient, energy, and oxygen requirements as well as for removal of metabolic waste. Cancer cells within a tumour generate such pathologic vasculature by recruiting endothelial cells to the tumour site. This is accomplished by secreting molecular factors, such as the well-known vascular endothelial growth factor (VEGF, into the extracellular space.

VEGF binding to VEGF receptors on endothelial cells results in the migration and recruitment of endothelial cells. In this way, proteins expressed by cancer cells can regulate the cellular and structural content of tumours—giving rise to continued tumour growth. Recent work has revealed a major role for another class of genes— known as small non-coding RNAs (microRNAs)—in the regulation of endothelial recruitment and tumour angiogenesis.

One member of this family (miR-126) was recently found to inhibit endothelial recruitment by suppressing a set of cancer genes that activate endothelial migration. In this way, a non-coding RNA expressed by cancer cells could shape the tumour and metastatic microenvironment.

This is thus depicted below from the Halberg paper. Here we have two cells, the top cell is a cancer cell where miR126 is blocking IGBP2 and blocking the MERTK receptor which in turn would have blocked the entrance of GAS6. But since miR126 has blocked the blocker, we have excess GAS6. Thus we have a problem, namely the GAS6 "overproduction" is really a failure to block resulting from the cancer cell miR126 production.



A small non-coding RNA, miR-126, suppresses endothelial migration by targeting a set of genes in cancer cells that drive endothelial migration. These genes drive endothelial migration by activating endothelial IGF1 receptor, a promoter of endothelial migration, and inhibiting endothelial MERTK receptor, a suppressor of endothelial migration. Mertk ECD, Mertk extracellular domain.

From: Halberg et al, EMBO 2012

It is critical always therefore to look across all paths, direct as well as epigenetic.

As Zhuang et al state:

Angiogenesis plays a crucial role during tumorigenesis and much progress has been recently made in elucidating the role of VEGF and other growth factors in the regulation of angiogenesis. Recently, microRNAs (miRNAs) have been shown to modulate a variety of physiological and pathological processes.

We identified a set of differentially expressed miRNAs in microvascular endothelial cells co-cultured with tumour cells. Unexpectedly, most miRNAs were derived from tumour cells, packaged into microvesicles (MVs), and then directly delivered to endothelial cells.

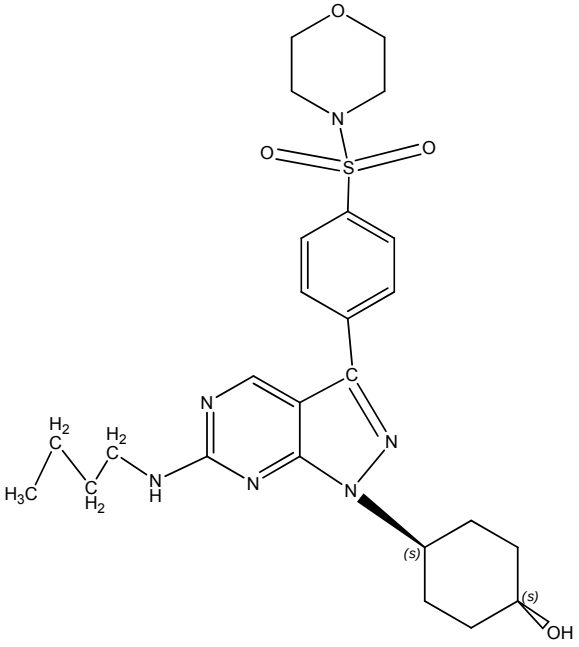
Among these miRNAs, we focused on miR-9 due to the strong morphological changes induced in cultured endothelial cells. We found that exogenous miR-9 effectively reduced SOCS5 levels, leading to activated JAK-STAT pathway. This signalling cascade promoted endothelial cell migration and tumour angiogenesis.

Remarkably, administration of anti-miR-9 or JAK inhibitors suppressed MV-induced cell migration in vitro and decreased tumour burden in vivo. Collectively, these observations suggest that tumour-secreted miRNAs participate in intercellular communication and function as a novel pro-angiogenic mechanism.

5.3.5 Inhibitors

Inhibitors of pathways are being developed at a rapid rate. Knowing the pathway and molecular structure of the receptors it is somewhat readily possible to develop a strong inhibitor, a molecule that interferes with the normal ligand.

Schlegel et al have developed and tested an inhibitor of the MERTK receptor and it is shown below.



Schlegel et al characterize this molecule as follows:

A novel MERTK tyrosine kinase inhibitor, UNC1062, inhibits MERTK mediated signaling, promotes apoptosis, and inhibits colony formation in melanoma cells. While activating mutations in BRAF and NRAS occur in melanoma at rates of 41% and 18%, respectively, lower mutation frequency or gene amplifications in other signaling molecules, such as RTKs, can also contribute to melanoma pathogenesis.

UNC1062 was developed as a MERTK-selective tyrosine kinase inhibitor. Its structure is based on a previously published pyrazolopyrimidine scaffold, and it has an improved affinity and specificity profile compared with its parent compound, UNC569 .

UNC1062 potently inhibits MERTK kinase activity in vitro and exhibits specificity within the TAM family. Treatment of HMCB and G361 cells with increasing concentrations of UNC1062 resulted in a potent dose-dependent reduction in MERTK phosphorylation

In the work of Verma et al they present an interesting collection of molecules which exhibit inhibitor characteristics (see their Figure 2). This is an expansion of what Schlegel et al have presented.

Again from Schlegel et al we have:

MAPK/ERK and PI3K/AKT are 2 of the most frequently dysregulated pathways in melanoma. These 2 pathways not only play a role in melanoma development and progression, but are also involved in primary and secondary resistance to BRAF inhibitors.

The observation that MERTK signals via both pathways, as well as through others whose roles in melanoma biology are currently unclear (e.g., STAT6), not only highlights the complex regulation of these pathways by membrane receptors, such as MERTK, but may also provide a therapeutic advantage, since targeting MERTK may disrupt signaling in multiple pathways.

These observations and the data presented here suggest that MERTK-targeted therapies could potentially be considered for patients, irrespective of BRAF and NRAS status and/or prior treatment with BRAF inhibitors.

The latter observation is of possible significant merit. Namely the MERTK targeting allows for alternative pathway blocking, namely doing so at the source of pathway activation.

5.3.6 Observations

We conclude with some general and specific observations. This work by Schlegel et al is of significant importance for reasons already indicated.

1. **MERTK presents an attractive target for metastatic diseases.**

To best summarize, we use the words directly from the paper. Schlegel et al conclude:

We believe this work has led to several novel insights.

First, MERTK expression is significantly elevated in distant metastatic tumors compared with primary melanomas.

Second, MERTK is overexpressed in approximately half of melanoma cell lines, irrespective of BRAF and NRAS status, and is an active receptor.

Third, targeting MERTK suppresses prosurvival pathways such as STAT6, AKT, and ERK1/2.

Fourth, targeting MERTK suppresses colony-forming potential and migration.

And fifth, targeting MERTK in vivo retards tumor growth in a human melanoma xenograft model.

The finding that MERTK expression is highest in distant metastatic melanomas compared with primary melanomas and the roles of MERTK in colony formation, migration, and invasion suggest that MERTK plays a role in the progression of primary melanomas and the development of distant metastases.

Similar to the observations in this report, the migratory nature of glioblastoma cells could be reduced by MERTK inhibition with either shRNA knockdown or a MERTK monoclonal antibody, suggesting that increased MERTK expression may contribute to outgrowth of the metastatic tumor.

2. **MER and other TAM receptors show significant impact across broad areas of cancer activity.**

Now Verma et al present an interesting summary table as show below which recounts what cancer types are also upregulated TAM pathways. The breath of such upregulation is significant. It also may present significant opportunities for blockage molecules, namely inhibitors of the total pathway.

Cancer type	Upregulation of Axl/Mer/Gas
Acute leukemia (ALL, AML)	Axl, Mer
Astrocytoma	Axl, Mer, Gas 6
Breast cancer	Axl, Mer, Gas 6
Colorectal carcinoma	Axl
Esophageal adenocarcinoma	Axl
Gastrointestinal stromal tumors	Axl
Gastric cancer	Axl, Mer, Gas 6
Hepatocellular carcinoma	Axl
Kaposi sarcoma	Axl

Lung cancer	Axl
Mantle cell lymphoma	Mer
Melanoma	Axl, Mer
Ovarian cancer	Axl, Gas 6
Osteosarcoma	Axl
Pancreatic ductal adenocarcinoma	Axl, Gas 6
Renal cell carcinoma	Axl, Gas 6
Prostate cancer	Axl, Mer
Thyroid cancer	Axl, Gas 6
Uterine endometrial cancer	Axl, Gas 6

3. **GAS6 Inhibition on MERTK by inhibitors is an attractive approach to metastatic melanoma**

Developing receptor inhibitors is a powerful approach to controlling metastatic growth and proliferation.

As Verma et al state:

A potential ability of sAxl to serve as a natural antagonist of Gas 6 could have clinical relevance. Similarly, the membrane-bound Mer protein is cleaved in the extracellular domain via a metalloproteinase (38). Further studies are needed to establish sAxl and sMer as important biomarkers for correlation with disease stage and predicting prognosis.

As Segal et al state:

A subset of genes, including the small monomeric GTPase RABB33, the proto-oncogene MERTK, the glycopeptide hormone STC1, and the neuropeptide GAL were shown to discriminate CCS/MSP from both STS and melanoma.

We further surveyed specific genes of interest and found melanoma differentiation antigens TYRP1, TYRP2/DCT, and MART-1 to be expressed at varying levels in the CCS/MSP specimens. PMEL17 was most consistently expressed in all four tumors in a similar distribution to that of MITF. Interestingly, SOX10, which induces MITF expression, was expressed in all CCS/MSP and most melanoma specimens

Thus it appears that this approach and ones like it are useful for thorough examination as attractive and effective means of metastatic control and management.

However there is still a long way from this point to approved therapeutics.

5.4 LIGANDS

Ligands are the intercellular proteins, gene products that cause cell modifications. They can attach and result in mitotic behavior as well as the increased production of other proteins. Ligands we have presented thus far include the following:

- EGF
- Hedgehog
- Her2
- HGF
- IGFBP7
- MAPK
- PDGF
- TGF
- VEGF
- Wnt

Many of these are classic growth factors, namely EGF, HGF, PDGF, TGF and VEGF. We shall not focus on them. The one of most significance are Wnt and Hedgehog. These two are in many ways also examples of how ligands function across the cell. Now DeVita et al relate the basis for IGFBP7 being recognized as a putative factor in melanoma. We leave that discussion to them.

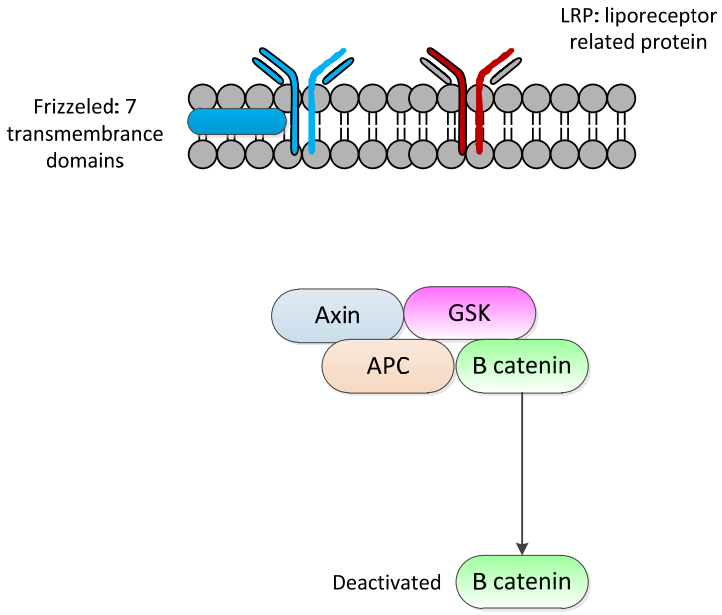
There are several other pathway elements that are worth commenting on. We do three here; WNT, Hedgehog, and Notched.

5.4.1 WNT

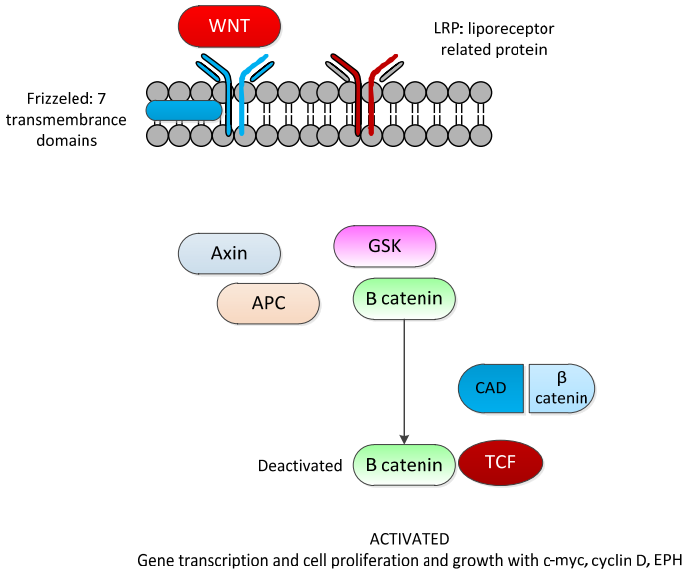
Wnt is characterized as follows:

- The name comes from the “wingless” gene and thus the Wn prefex. This was related to discovery on fruitflies.
- The **canonical Wnt pathway** describes a series of events that occur when Wnt proteins bind to cell-surface receptors of the Frizzled family, causing the receptors to activate Dishevelled family proteins and ultimately resulting in a change in the amount of β-catenin that reaches the nucleus
- Dishevelled (DSH) is a key component of a membrane-associated Wnt receptor complex which, when activated by Wnt binding, inhibits a second complex of proteins that includes axin, GSK-3, and the protein APC
- The axin/GSK-3/APC complex normally promotes the proteolytic degradation of the β-catenin intracellular signaling molecule.
- After this "β-catenin destruction complex" is inhibited, a pool of cytoplasmic β-catenin stabilizes, and some β-catenin is able to enter the nucleus and interact with TCF/LEF family transcription factors to promote specific gene expression

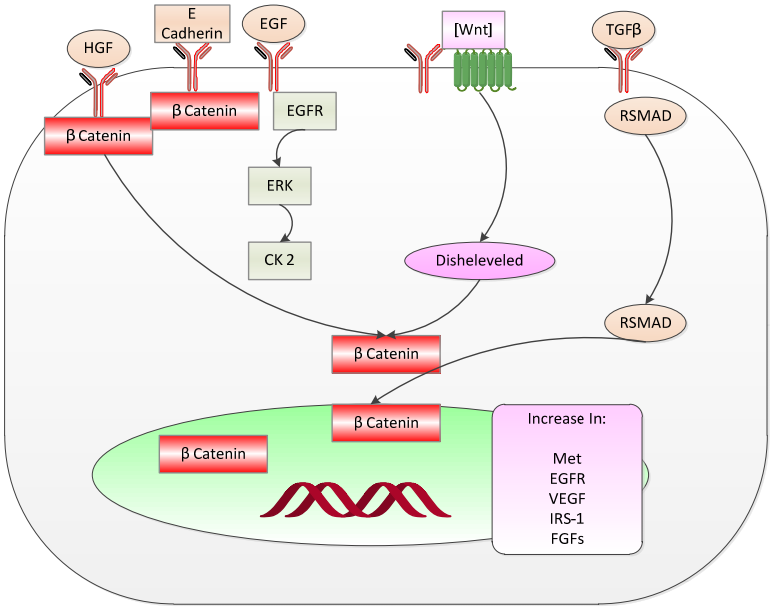
We depict this below, first the inactive state:



Then we depict the activated state with Wnt attached to the receptor:

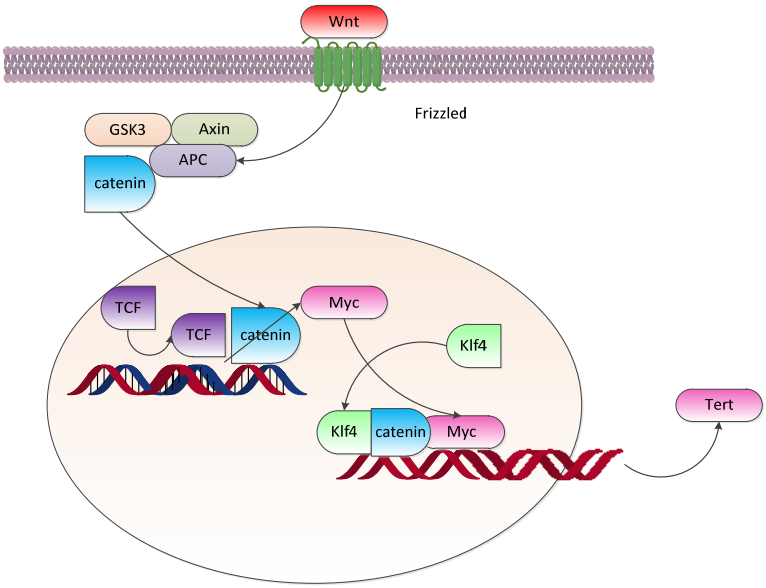


The figure below details many of the effects of Wnt binding:



5.4.2 Wnt and TERT Signaling

Signaling pathways in the cells have been a major focus on study for the past decade or so. The focus generally has been on what protein or gene influences what other protein or gene. A recent article by Greider presents some interesting work on Wnt and TERT[71].



[71] Greider, Wnt Regulates TERT—Putting the Horse Before the Cart, SCIENCE VOL 336 22 JUNE 2012, p 1519.

Wnt is an extra cellular signaling protein and it attaches to Frizzled a receptor and sets off a cascade that moves B catenin into the nucleus and generates Myc which is a transcription protein with together with catenin and other transcription proteins generates Tert from TERT.

To quote from NCBI[72]:

Telomerase is a ribonucleoprotein polymerase that maintains telomere ends by addition of the telomere repeat TTAGGG. The enzyme consists of a protein component with reverse transcriptase activity, encoded by this gene, and an RNA component which serves as a template for the telomere repeat. Telomerase expression plays a role in cellular senescence, as it is normally repressed in postnatal somatic cells resulting in progressive shortening of telomeres. Deregulation of telomerase expression in somatic cells may be involved in oncogenesis.

As the Science article states:

Maintaining the length of telomere, the ends of chromosomes, is essential for all cells that divide many times. The enzyme telomerase lengthens these ends, counterbalancing their shortening that occurs each time chromosomes are copied. Telomerase is essential for cell viability, and loss of its function from the loss of only one of two copies of the encoding gene can lead to the failure of stem cell renewal that is seen in premature aging conditions such as dyskeratosis congenita, aplastic anemia, and pulmonary fibrosis. Conversely, telomerase activity is increased in many cancers and may be required for cancer cells to maintain their telomere length...

They continue is a rather interesting wording:

Because of the importance of telomerase expression, the signaling pathways that control TERT transcription have been extensively studied. Remarkably, many different transcription factors, including c-Myc, Sp1, nuclear factor of activated T cells (NFAT), activating protein 2B, nuclear factor κB (NF-κB), Myb, activating transcription factor, nuclear factor 1 (NF1), and the estrogen receptor (ER), bind to the 330–base pair minimal TERT promoter and regulate transcription. In addition, a number of negative regulators bind the TERT promoter, including CTCF, elongation factor 2, p53, Ets, Mad1, Men1, and Wt1. Adding β-catenin and Klf4 to the many regulators that bind the TERT promoter is like adding one more guest to a crowded table at a dinner party.

They conclude:

It is reasonable to propose that Wnt regulates TERT given that Wnt signaling plays an essential role in stem cell self-renewal and that TERT is needed for the long-term growth of stem cells. TERT regulation seems to require not one, but two master transcriptional regulators to assure that there is neither too much, which may allow the growth of cancer cells, nor too little, which might lead to stem cell failure. The finding by Hoffmeyer et al. that both β-catenin and Klf4 are required to activate TERT expression puts the horse (Wnt) before the cart (TERT) and provides a foundation for linking telomerase levels and self-renewal.

[72] http://www.ncbi.nlm.nih.gov/gene/7015

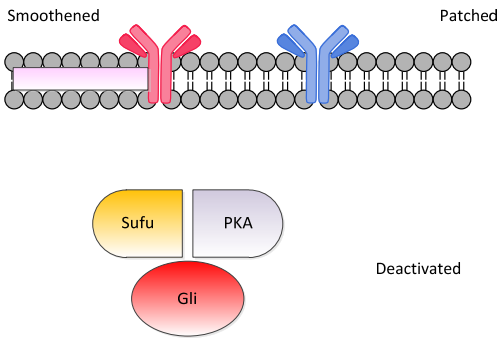
The observation of the inter-cellular signalling with Wnt and its control over TERT and the telomere process is quite interesting. This may be an interesting way to incorporate many of the Turing models we have been discussing as well.

5.4.3 Hedgehog

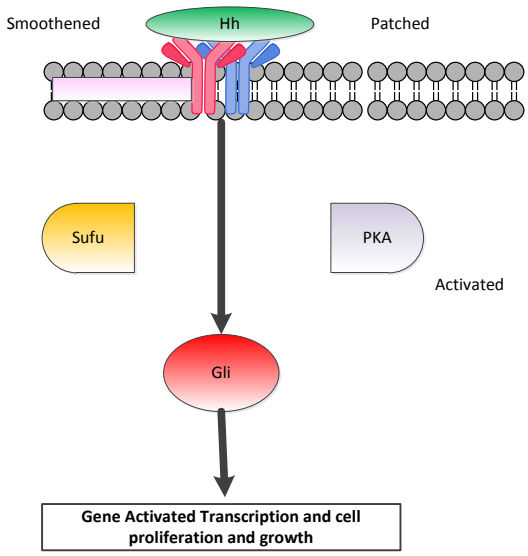
Hedgehog is a ligand which activates receptors and then pathways. The Hedgehog pathway is also a key element characterized as follows:

- In the absence of Hh a cell-surface transmembrane protein called Patched (PTCH) acts to prevent high expression and activity of a 7 membrane spanning receptor called Smoothened (SMO).
- Patched has sequence similarity to known membrane transport proteins. When extracellular Hh is present, it binds to and inhibits Patched, allowing Smoothened to accumulate and inhibit the proteolytic cleavage of the Ci protein.
- In cells with Hh-activated Patched, the intact Ci protein accumulates in the cell cytoplasm and levels of CiR decrease, allowing transcription of some genes such as decapentaplegic (dpp, a member of the BMP growth factor family).
- For other Hh-regulated genes, expression requires not only loss of CiR but also the positive action of uncleaved Ci acting as a transcriptional activator.

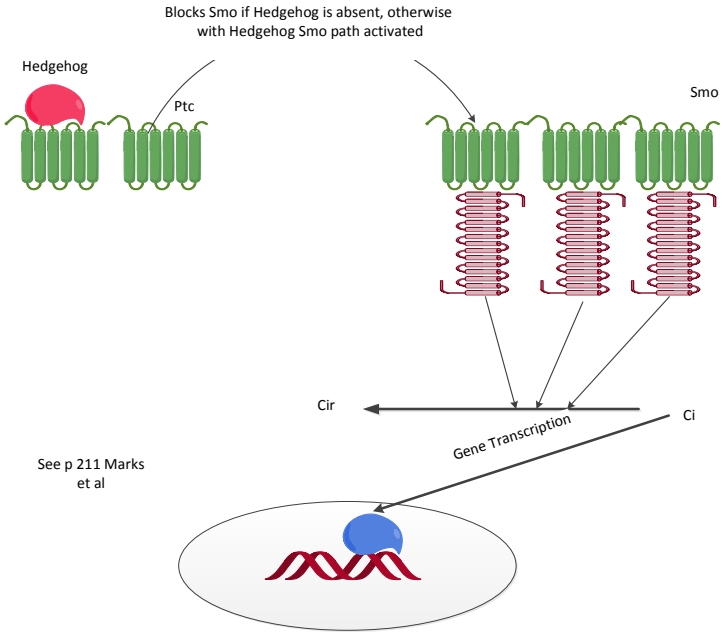
First we show it inactivated state as below. Note we have two separate receptors, Patched and Smoothened, which are separate and non-functional. Sufu and PKA are bound and Gli is also bound. Gli is the encoded transcription factor is activated by the sonic hedgehog signal transduction cascade and regulates stem cell proliferation. The activity and nuclear localization of this protein is negatively regulated by p53 in an inhibitory loop. Thus by activating Hh and combining the two receptors, Smo (Smoothened) and Patched (Ptch) we then activate Gli by unbinding it from Sufu and PKA. This is an example where we have three type at once; Wht the ligand, Smo and Ptch as receptors and Gli as a transcription factor.



Then the activated pathway as follows:

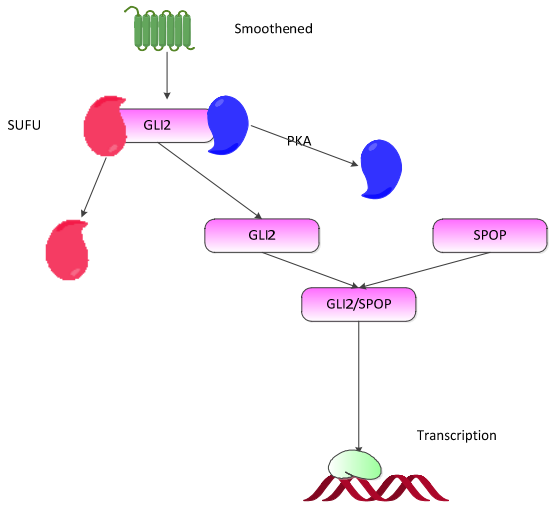


We demonstrate in more detail below the Hh binding. This graphically demonstrates the activation of the transcription factor and its movement into the nucleus and transcribing.



This is a first example. It demonstrates very simplistic terms of operation. Let us examine a bit more in detail. First, there may very well be many sets of receptors. The proximity demonstrated in the above raises the question of having Wnt being able to draw the two receptors together. It is not at all clear how that works. However from a systems perspective we shall assume it a fait accompli. Yet we cannot assume that we may very well have multiple sets, and thus multiple Gli released. That could then raise the rate of transcription. Modeling this level of complexity is essential. Also we have the issue of having an increase in transcription, so what? Having more proteins may or may not be a problem, it depends upon what proteins. These issues in detail are not readily examined at the bench level.

We demonstrate below the Smo, Smoothened, and activation in some further detail.



Note that we have shown additional detail on the pathway elements resulting in transcription. It should be noted that there is considerably more detail available but we shall try to keep this at a level adequate for a model.

5.4.4 Notched

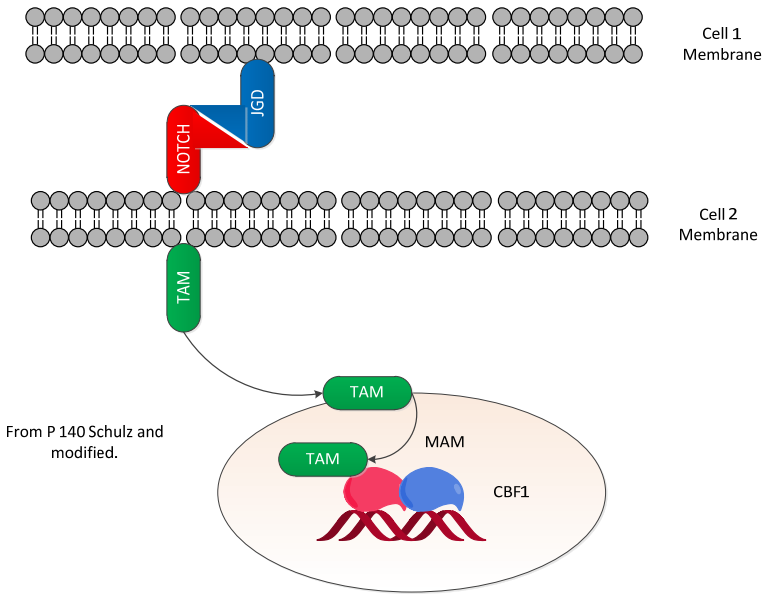
Notched is a bit of an amalgam of the above discussion. The notched pathway is characterized as follows.

The notch protein sits like a trigger spanning the cell membrane, with part of it inside and part outside. Ligand proteins binding to the extracellular domain induce proteolytic cleavage and release of the intracellular domain, which enters the cell nucleus to alter gene expression. The notch signaling pathway is important for cell-cell communication, which involves gene regulation mechanisms that control multiple cell differentiation processes during embryonic and adult life. Notch signaling also has a role in the following processes:

1. neuronal function and development
2. stabilization of arterial endothelial fate and angiogenesis
3. regulation of crucial cell communication events between endocardium and myocardium during both the formation of the valve primordial and ventricular development and differentiation
4. cardiac valve homeostasis, as well as implications in other human disorders involving the cardiovascular system
5. timely cell lineage specification of both endocrine and exocrine pancreas
6. influencing of binary fate decisions of cells that must choose between the secretory and absorptive lineages in the gut

7. expansion of the hematopoietic stem cell compartment during bone development and participation in commitment to the osteoblastic lineage, suggesting a potential therapeutic role for notch in bone regeneration and osteoporosis
8. T cell lineage commitment from common lymphoid precursor
9. regulation of cell-fate decision in mammary glands at several distinct development stages
10. possibly some non-nuclear mechanisms, such as control of the actin cytoskeleton through the tyrosine kinase Ab

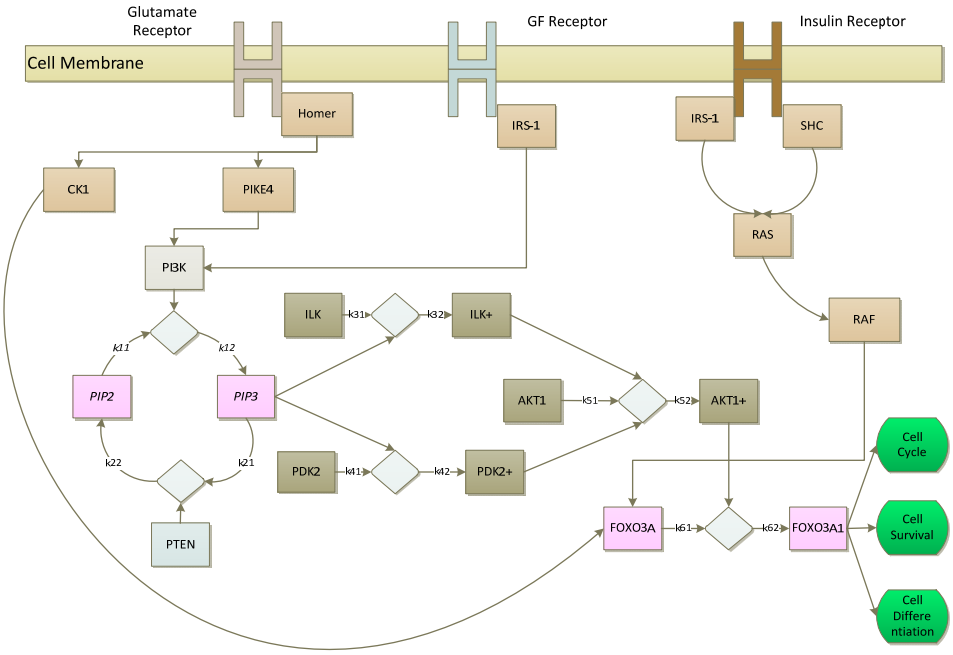
We demonstrate Notched and its counterpart Jagged in the following Figure. On the cell surface we have Notched and on the other cell surface we have Jagged. When they bond, in a sense as surface proteins but with a communicating capability, Notched release or activates Tam which is a transcription factor facilitator.



Notch signaling is dysregulated in many cancers.

5.4.5 Other Receptors

There are many other ligands and receptors and each has a property of activating pathways and in turn activating transcription agents. In the following Figure we depict three of additional merit; GR, IR, GFR. From each of these when activated we demonstrate the pathway crosstalk and activation.



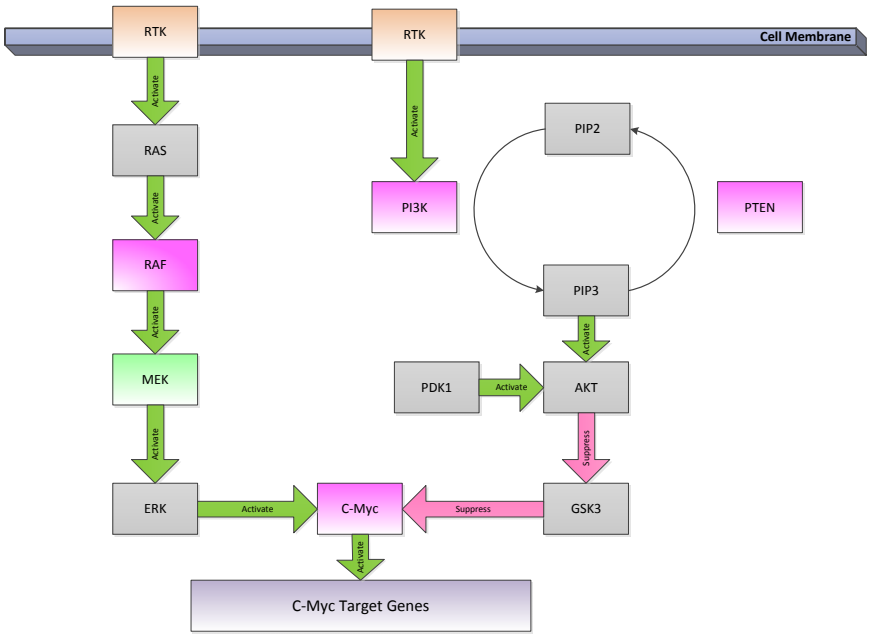
Note in the above we have demonstrated the move towards FOXO, a pathway element which activates cell growth, reproduction and transcription.

5.5 PATHWAY ELEMENTS

Now we can move on to pathways and their elements. Oftentimes the action in a malignant cell happens when a pathway element is compromised. Typical of such elements would be PTEN or p53. Loss of this functionality can cause significant loss in cell control. We shall examine several of the key path elements and place them in context with the other four major players. This will be a classification approach and will not endeavor to establish a full model.

5.5.1 Akt

AKT is gene whose product Akt is a key player in one of the major pathways in cell homeostasis. The figure below shows its position. We will see this Figure many times as we progress. It details two key pathways, the RAS/RAF/MEK/ERK on the left and the AKT/PTEN/PI3K on the right. Both eventually come to control the transcription factor c-Myc.



As LoPiccolo et al state:

Signaling through the PI3K/Akt/mTOR pathway can be initiated by several mechanisms, all of which increase activation of the pathway in cancer cells. Once activated, the PI3K/Akt/mTOR pathway can be propagated to various substrates, including mTOR, a master regulator of protein translation. Initial activation of the pathway occurs at the cell membrane, where the signal for pathway activation is propagated through class IA PI3K.

Activation of PI3K can occur through tyrosine kinase growth factor receptors such as epidermal growth factor receptor (EGFR) and insulin-like growth factor-1 receptor (IGF-1R), cell adhesion molecules such as integrins, G-protein-coupled receptors (GPCRs), and oncogenes such as Ras. PI3K catalyzes phosphorylation of the D3 position on phosphoinositides to generate the biologically active moieties phosphatidylinositol-3,4,5-triphosphate ($PI(3,4,5)P_3$) and phosphatidylinositol-3,4-bisphosphate ($PI(3,4)P_2$).

Upon generation, PI(3,4,and 5)P_3 binds to the pleckstrin homology (PH) domains of PDK-1 (3_-phosphoinositide-dependent kinase 1) and the serine/threonine kinase Akt, causing both proteins to be translocated to the cell membrane where they are subsequently activated. The tumor suppressor PTEN (phosphatase and tensin homolog deleted on chromosome 10) antagonizes PI3K by dephosphorylating PI(3,4,and 5)P_3 and ($PI(3,4)P_2$), thereby preventing activation of Akt and PDK-1.

As Miller and Mihm state:

A second chromosomal region that is frequently affected by homozygous deletion in melanoma and other cancers is the PTEN locus on chromosome 10.

PTEN encodes a phosphatase that attenuates signaling by a variety of growth factors that use phosphatidylinositol phosphate (PIP3) as an intracellular signal. In the presence of such growth factors, intracellular levels of PIP3 rapidly increase. This increase triggers the activation of protein kinase B (PKB, also called AKT) by phosphorylation.

Activated AKT phosphorylates and inactivates proteins that suppress the cell cycle or stimulate apoptosis, thereby facilitating the proliferation and survival of cells. PTEN normally keeps PIP3 levels low; in its absence, levels of PIP3 and active (phosphorylated) AKT increase.

Increased AKT activity prolongs cell survival through the inactivation of BCL-2 antagonist of cell death (BAD) protein and increases cell proliferation by increasing CCND1 expression, and affects many other cell-survival and cell cycle genes through the activation of the forkhead (FKHR) transcription factor.

AKT activity can also be increased in cells by mutations that cause the amplification and over-expression of the protein. Restoration of PTEN in cultured mouse melanocytes decreases the ability of the cells to form tumors. In model systems, suppression of AKT3, a member of the AKT family, reduces the survival of melanoma cells and the growth of human melanomas implanted in immunodeficient nude mice…

Akt has played a role in various examinations or melanoma related genes. Moreover as Stahl et al state:

Malignant melanoma is the skin cancer with the most significant impact on man, carrying the highest risk of death from metastasis. Both incidence and mortality rates continue to rise each year, with no effective long-term treatment on the horizon.

In part, this reflects lack of identification of critical genes involved and specific therapies targeted to correct these defects.

We report that selective activation of the Akt3 protein promotes cell survival and tumor development in 43 to 60% of non-familial melanomas. The predominant Akt isoform active in melanomas was identified by showing that small interfering RNA (siRNA) against only Akt3, and not Akt1 or Akt2, lowered the amount of phosphorylated (active) Akt in melanoma cells. The amount of active Akt3 increased progressively during melanoma tumor progression with highest levels present in advanced-stage metastatic melanomas.

Mechanisms of Akt3 deregulation occurred through a combination of overexpression of Akt3 accompanying copy number increases of the gene and decreased PTEN protein function occurring through loss or haplo-insufficiency of the PTEN gene.

Targeted reduction of Akt3 activity with siRNA or by expressing active PTEN protein stimulated apoptotic signaling, which reduced cell survival by increasing apoptosis rates thereby inhibiting melanoma tumor development. Identifying Akt3 as a selective target in melanoma cells provides new therapeutic opportunities for patients in the advanced stages of this disease.

The work by Stahl et al identifies a variant of Akt as causative in the development of melanoma. Just as importantly they identify a siRNA as part of the process. We shall be discussing this as part of our discussion on epigenetic factors.

5.5.2 PI3K

As Davies states:

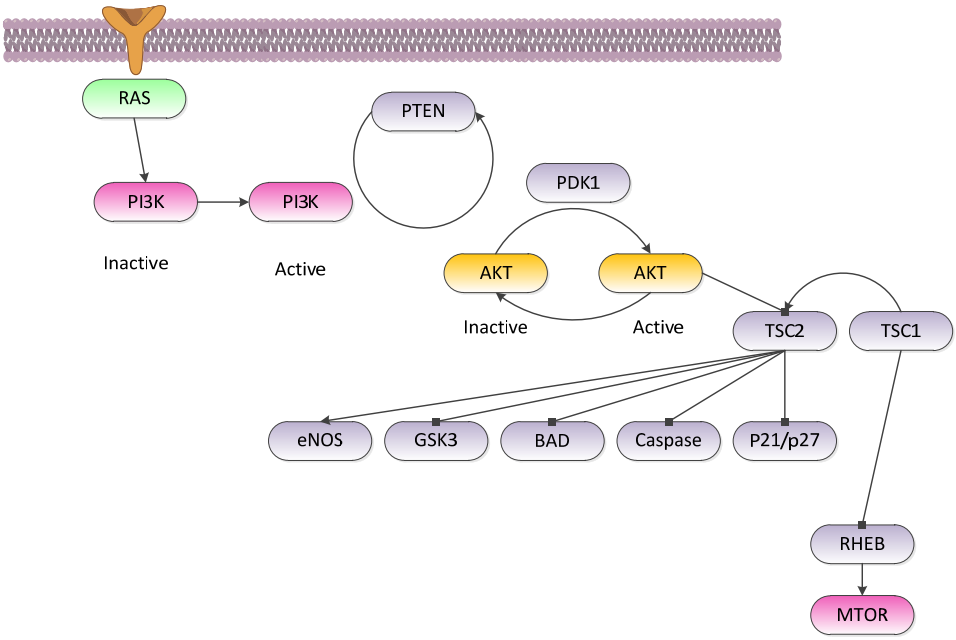
The PI3K (phosphatidylinositol 3-kinase)/AKT pathway is one of the most important signaling networks in cancer. There is growing evidence that activation of this pathway plays a significant role in melanoma, frequently in the setting of concurrent activation of RAS-RAF/MEK- ERK signaling. This evidence includes the identification of genetic and epigenetic events that activate this pathway in melanoma cell lines and clinical specimens. In addition, functional experiments have demonstrated important roles for the PI3K-AKT pathway in both melanoma initiation and therapeutic resistance. The availability of many inhibitors against the PI3K-AKT pathway is rapidly leading to the development of trials that will ultimately determine its clinical significance in this disease. The rational development of such therapies will be facilitated by strategies that utilize the growing understanding of the complexity of the regulation and roles of this pathway.

The PI3K (phosphatidylinositol 3-kinase)YAKT pathway is a critical regulator of many essential cellular processes. In addition to playing an important role in normal cellular physiology, activation of PI3K-AKT signaling is one of the most frequent events in cancer.[9] There is growing evidence that the PI3K-AKT pathway is frequently activated in melanomas and plays a functionally important role in this disease. The availability of multiple inhibitors against this pathway and recent insights into rational approaches to target it make understanding the role of PI3K-AKT activation in melanoma clinically important.

Melanoma tumors and cell lines with loss of PTEN generally demonstrate high levels of activation of the PI3K-AKT pathway. PTEN *gene mutations and deletions are mutually exclusive with activating* NRAS *mutation in melanoma.41 In contrast, many melanomas with loss of PTEN have concurrent activating BRAF mutations.[42,43] This genetic interaction between BRAF and PTEN has been demonstrated functionally in mouse models.*

Genetic, expression-based, and functional data strongly support that the PI3K-AKT pathway likely plays an important role in at least a subset of melanomas. However, there are still several gaps in the understanding of this pathway in this disease. Whereas translational and functional studies of the PI3K-AKT pathway have generally examined cutaneous melanomas, its role in other melanoma subtypes is less well defined. In addition, there are limited data at this point regarding the concordance of PI3K-AKT pathway aberrations and status among different tumors in individual patients. Although are several studies supporting a high concordance of activating BRAF and NRAS mutations in melanoma patients, data in other tumors types suggest that the PI3KAKT pathway could be more variable

Davies demonstrates the linkages in the following:



The above is Davies description of the PI3K/AKT pathway activation.

Davies concludes with:

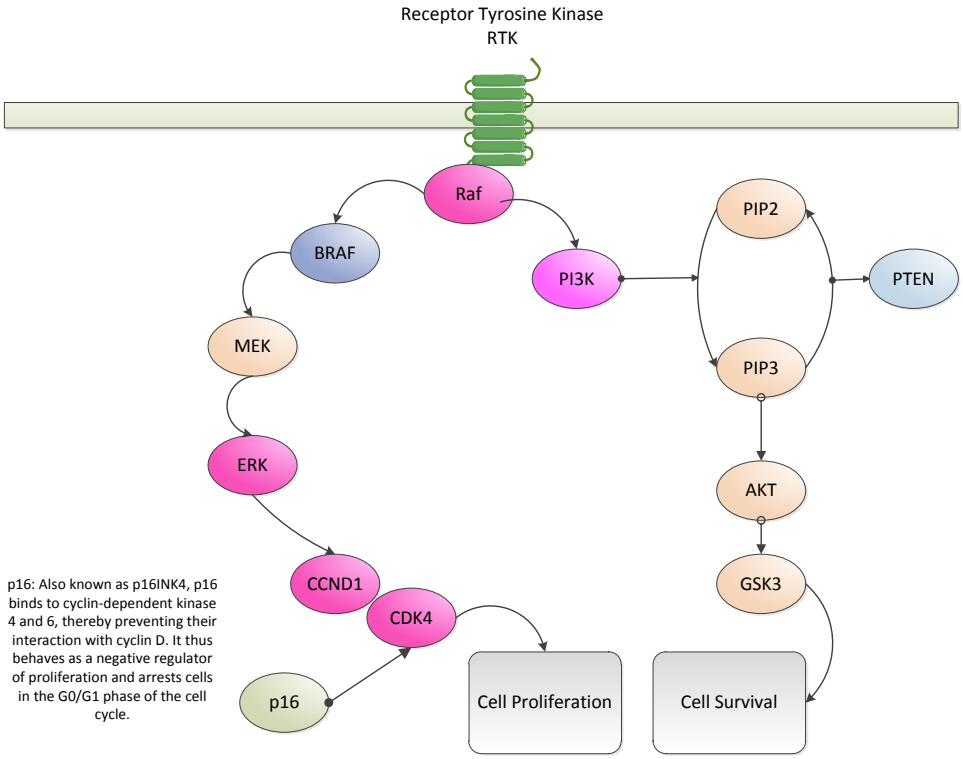
The high prevalence of activating BRAF and NRAS mutations in melanoma provided a strong rationale to test the effects of inhibitors against the RAS-RAF-MEK-ERK pathway in this disease. Multiple studies demonstrated that inhibition of the BRAF protein in human melanoma cell lines with small inhibitory RNA or small molecules inhibited the in vitro growth of melanoma cell lines with activating BRAF mutations.[45] In particular, second-generation BRAF inhibitors, such as vemurafenib (also known as PLX4032) and GSK2118436 (also known as dabrafenib), which have a higher affinity for V600-mutant BRAF proteins than wild-type BRAF, induced apoptosis in BRAF-mutant melanoma cell lines and xenograft regression in animalmodels.

Treatment with vemurafenib or GSK2118436 resulted in confirmed clinical responses (by RECIST criteria) in 50% of metastatic melanoma patients with BRAF V600E mutations in clinical trials; in contrast, no patients with a wildtype BRAF responded.[1,47,48] Preclinical characterization of MEK inhibitors also demonstrated a positive correlation between the presence of activating BRAF mutations and sensitivity to these agents.[49] Promising clinical results have also been observed with the MEK inhibitor GSK1120212 in melanoma patients with BRAF mutations.

5.5.3 RAF

The most critical RAF gene, B-RAF, has been identified as a major player in the development of melanoma. This is also the target for the first genetic control of the malignancy. Thus we shall focus on B-RAF and its impact.

The Figure below demonstrates this character of B-RAF. The RTK, receptor Tyrosine Kinas can get activated and Raf activates B-RAF which in turn activates MEK and then ERK then CCD1 along with CDK4, which then ensures cell proliferation through mitotic process. Note again on the right the PI3K/AKT/GSK3 pathway which manages cell survival. Between these two processes we have a guaranteed means for managing metastatic growth.



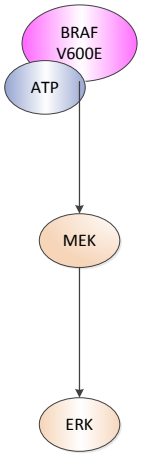
As Babchia et al state:

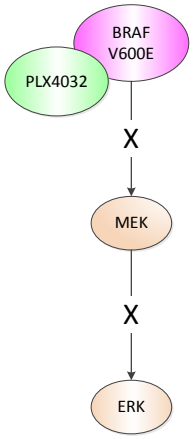
Activated PI3K/Akt attenuates the inhibitory effects of rapamycin on cell proliferation and thus serves as a negative feedback mechanism. This finding suggests that rapamycin is unlikely to inhibit uveal melanoma growth. In contrast, targeting PI3K while inhibiting B-Raf/ERK may be a promising approach to reduce the proliferation of uveal melanoma cells. … There is evidence that activation of mTOR inhibits PI3K/ Akt in some circumstances: rapamycin-induced inhibition of mTOR may enhance PI3K activation by an mTOR-dependent negative feedback mechanism for PI3K/Akt activation, at least in a few types of cells. Paradoxically, then, rapamycin, which

inhibits mTOR/p70S6K-mediated cell proliferation signaling, concurrently increases Akt phosphorylation and thus increases cell survival and proliferation. We speculated that this might be the case for uveal melanoma cells.

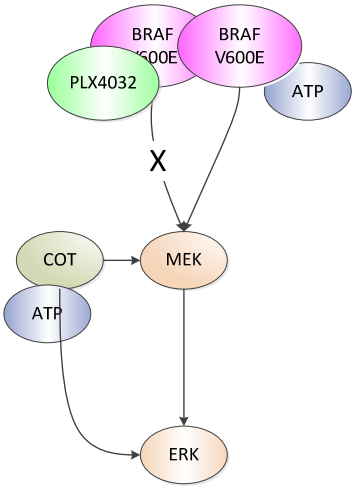
Analysis of Akt phosphorylation levels in rapamycin-treated cells showed that inhibition of mTOR greatly increased Akt phosphorylation without affecting Akt levels in uveal melanoma cell lines. This phenomenon was observed at a concentration as low as 10 nM rapamycin. …If our hypothesis about the mTOR feedback mechanism on PI3K/Akt activation is correct, PI3K inhibition would reduce the rapamycin-induced phosphorylation of Akt.

Thus Akt plays a role but it is ancillary as understood at this time. There





Note: PLX4032 replaces the activating ATP and deactivates the V600E BRAF.



From Solit and Rosen Fig 1: *"the overexpression of RAF1 or the activation of RAS as a result of RAS mutation or upstream activation of a receptor tyrosine kinase promotes:*

(i) the formation of RAF dimers. In cells expressing RAF dimers, binding of RAF inhibitors to one member of the dimer transactivates the other, nonbound member.

(ii) In such cells, PLX4032 does not inhibit MAP kinase signaling, which leads to drug resistance.

(iii) Alternatively, the overexpression of mitogen-activated protein kinase kinase kinase 8 (MAP3K8, or COT) results in RAF-independent activation of MEK and ERK and thus resistance to PLX4032.

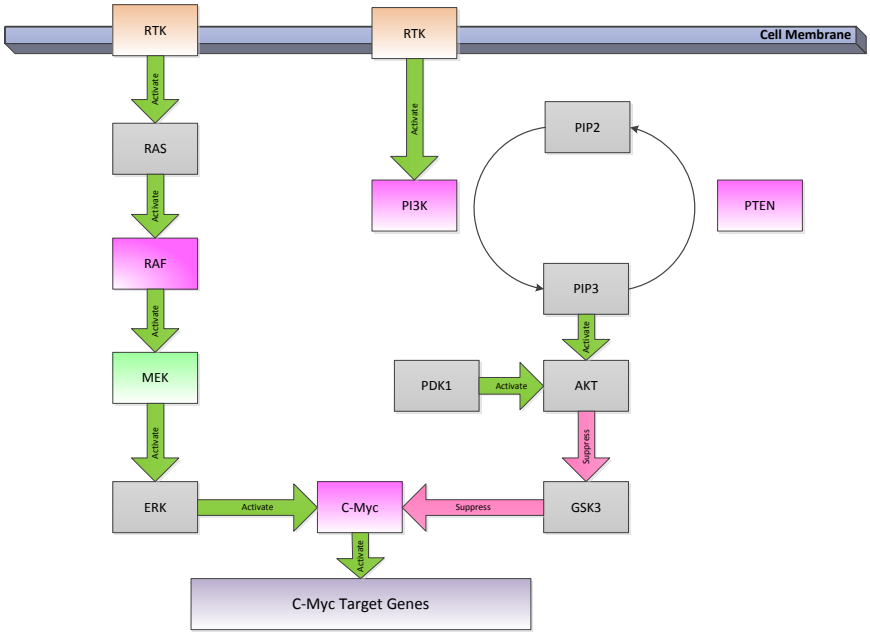
(iv) The activation of upstream receptor tyrosine kinases may also cause resistance to PLX4032 by activating RAS, as well as by activating parallel signaling pathways, which results in diminished dependence of the cell on RAF signaling. PDGFRβ denotes platelet-derived growth factor receptor β, and RAS-GTP RAS in its active, GTP-bound state."

5.5.4 PTEN

PTEN is a significant gene which controls the Akt pathway which in turn controls the replication of cells. Loss of PTEN is often seen in metastatic prostate cancer. In many ways it is the hallmark of this change. As stated in NCBI[73]:

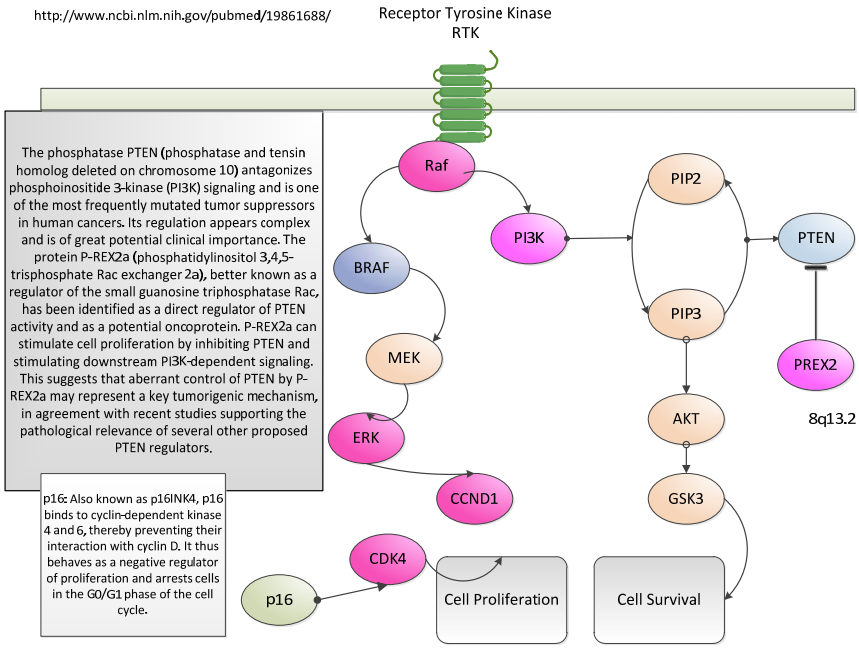
This gene was identified as a tumor suppressor that is mutated in a large number of cancers at high frequency. The protein encoded this gene is a phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase. It contains a tensin like domain as well as a catalytic domain similar to that of the dual specificity protein tyrosine phosphatases. Unlike most of the protein tyrosine phosphatases, this protein preferentially dephosphorylates phosphoinositide substrates. It negatively regulates intracellular levels of phosphatidylinositol-3,4,5-trisphosphate in cells and functions as a tumor suppressor by negatively regulating AKT/PKB signaling pathway.

First the PTEN pathway as shown below:

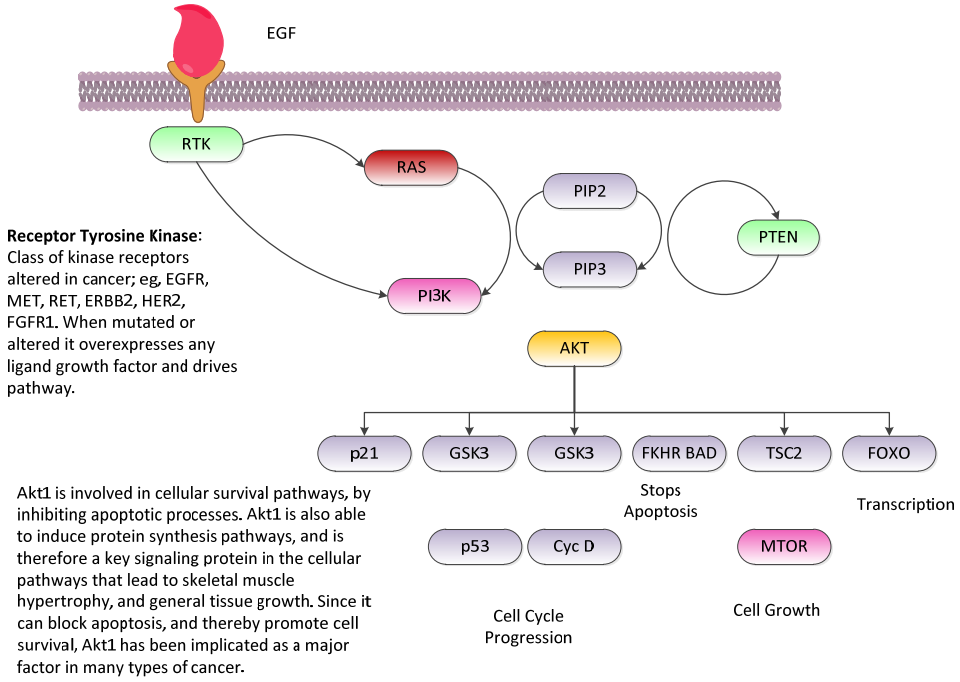


Note PTEN modulates the production of Akt which in turn modulates c-Myc which in turn controls cell reproduction. Any effect which causes PTEN to not be expressed will in turn result in unfettered cell growth.

[73] http://www.ncbi.nlm.nih.gov/gene/5728



PTEN has become a key gene in the development of prostate cancer. It controls a pathway leading up to c-myc control and once PTEN is lost the PCa can be considered as very aggressive. Its loss results in an activation of Akt and then c-myc causing uncontrolled cell growth. The pathway is shown below:



As Jelovac and Park state[74]:

The phosphatase and tensin homolog gene (PTEN) is a tumor suppressor located on the human chromosome 10q arm and is an important mediator of carcinogenesis in a variety of human malignancies. By the strictest definition, a tumor suppressor is a gene whose loss confers an increased lifetime risk of developing tumors. The most illustrative examples of genes that fulfill this criterion are those associated with familial cancer syndromes whereby heritable inactivation of 1 allele and subsequent increased tumor risk is passed along to each generation in an autosomal-dominant fashion.

Using this as a framework, PTEN is a bona fide tumor suppressor gene in that heritable germline mutations have been described in Cowden syndrome (CS), giving rise to a number of human tumors and cancers, most notably thyroid and breast cancers. As is the paradigm of tumor suppressor genes, affected patients with CS inherit 1 mutant inactive copy of PTEN from either parent, and the ensuing loss of the second allele results in tumor formation with subsequent genetic events that eventually lead to cancer. Although there are notable exceptions to this model, most heritable cancer syndromes are believed to adhere to this pattern.

From the work of McMenamin et al we have the slides below. Here is a case where PIN is still expressing PTEN but as we increase the grade of PCa we see the elimination of PTEN expression. Thus we can say that PIN is a state prior to PTEN suppression and a corollary may be that PCa aggressiveness is reflective of loss of PTEN and activation of Akt pathway.

5.5.5 Cyclin D

Cyclin D is one of the key regulators of the cell cycle. As Bunz states (Bunz, pp 218-221) the cell cycle goes through several well-known phases. There are phase specific kinases which are cyclins which are called that because they were found to increase or decrease in a cyclical manner as the cell cycle phase progressed.

In the cycles the cyclin binds with a cyclin-dependent kinas or CDK. The activated cyclin-CDK complex phosphoralates phase specific substrates. Cyclin D along with CDK4 and CDK6 facilitate the transition through G1 to the start of S for example. Cyclin E with CDK2 facilitates the transition from G1 to S. Cyclin A with CDK2 moves through S. Cyclin A/B with CDK1 moves through G2. Thus activation of Cyclin D is a sign that cell replication has commenced.

As stated in NCBI[75]:

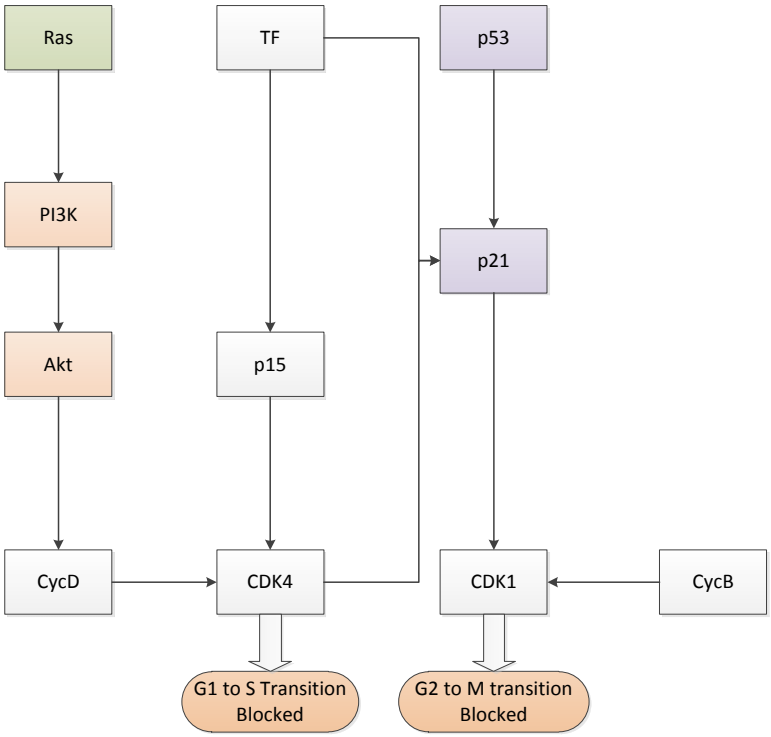
The protein encoded by this gene belongs to the highly conserved cyclin family, whose members are characterized by a dramatic periodicity in protein abundance throughout the cell cycle. Cyclins function as regulators of CDK kinases. Different cyclins exhibit distinct expression and degradation patterns which contribute to the temporal coordination of each mitotic event. This cyclin forms a complex with and functions as a regulatory subunit of CDK4 or CDK6, whose activity is required for cell cycle G1/S transition. This protein has been shown to interact with

[74] http://jama.ama-assn.org/content/304/24/2744.full

[75] http://www.ncbi.nlm.nih.gov/gene/595

tumor suppressor protein Rb and the expression of this gene is regulated positively by Rb. Mutations, amplification and overexpression of this gene, which alters cell cycle progression, is observed frequently in a variety of tumors and may contribute to tumorigenesis

Now we can look more closely at Cyclin D, CycD, as we show below. This we show as follows:



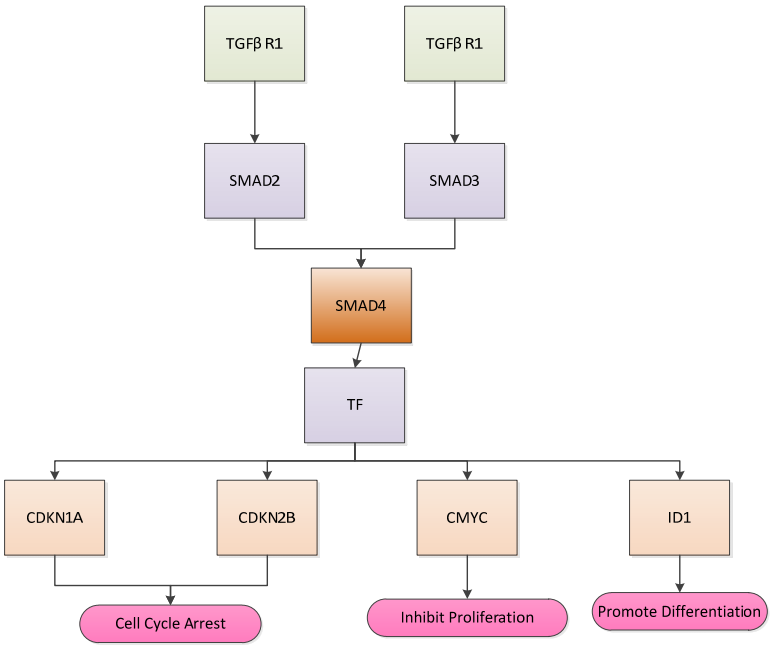
Note that Cyc D if not regulated will in turn fail to regulate the blocking of the G1 to S transition.

5.5.6 SMAD4

SMAD4 is an element in the TGF-β signalling chain. TGF is a cytokine, specifically a transforming growth factor cytokine. Like the Wnt-Apc pathway, the TGF pathway links defective development to cancer. The pathway is shown in part below (from Bunz p 199). Normal TGF signalling down-regulates the growth of most normal cells. Several of the genes in the TGF/SMAD pathway activation suppress growth. Specifically the genes CDKN1A and CDKN2B encode the cyclin dependent kinase inhibitors which suppress growth. Activated SMAD pathways also appear to suppress the transcription of other genes including c-Myc.

Gene	*Function*	*Disease*	*Pathway*
EWSR1	Translocation	Ewing's sarcomas, lymphomas, leukemias	SMAD
RUNX1	Translocation	Leukemias	SMAD
SMAD2	Inactivating codon change	Colon, breast	SMAD
TGFBR1, TGFBR2	Inactivating codon change	Colon, stomach, ovarian	SMAD

We show some of the TGF SMAD signalling below. We will elaborate this later.



SMAD4 controls the G1 to S transition. As stated in NCBI[76]:

This gene encodes a member of the Smad family of signal transduction proteins. Smad proteins are phosphorylated and activated by transmembrane serine-threonine receptor kinases in response to TGF-beta signaling. The product of this gene forms homomeric complexes and heteromeric complexes with other activated Smad proteins, which then accumulate in the nucleus and regulate the transcription of target genes.

This protein binds to DNA and recognizes an 8-bp palindromic sequence (GTCTAGAC) called the Smad-binding element (SBE). The Smad proteins are subject to complex regulation by post-translational modifications. Mutations or deletions in this gene have been shown to result in pancreatic cancer, juvenile polyposis syndrome, and hereditary hemorrhagic telangiectasia syndrome.

We use the NCI data set for its pathway[77]:

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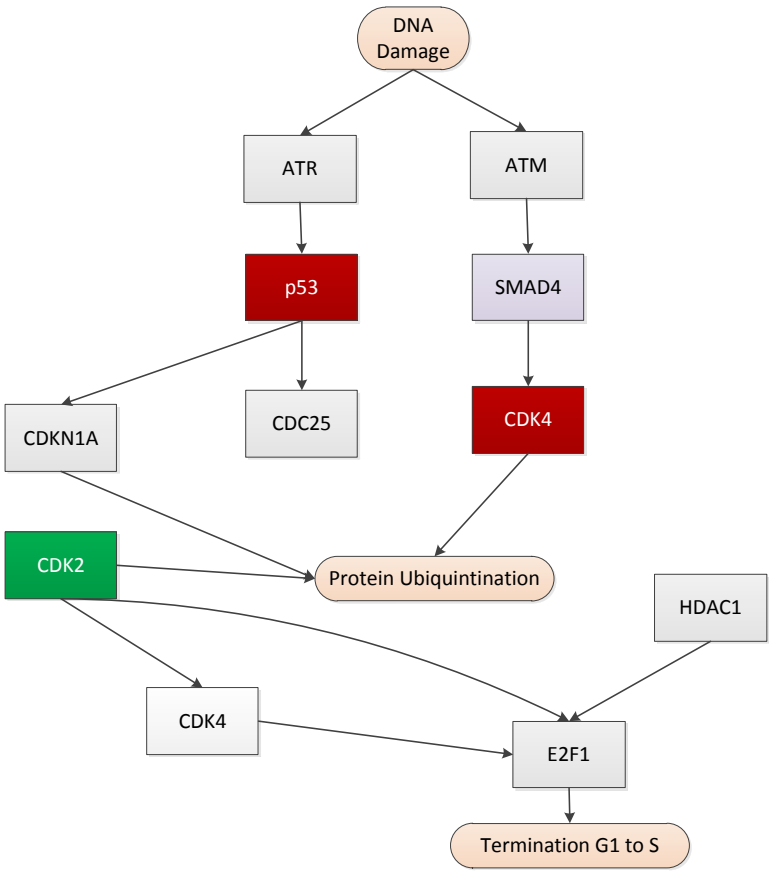
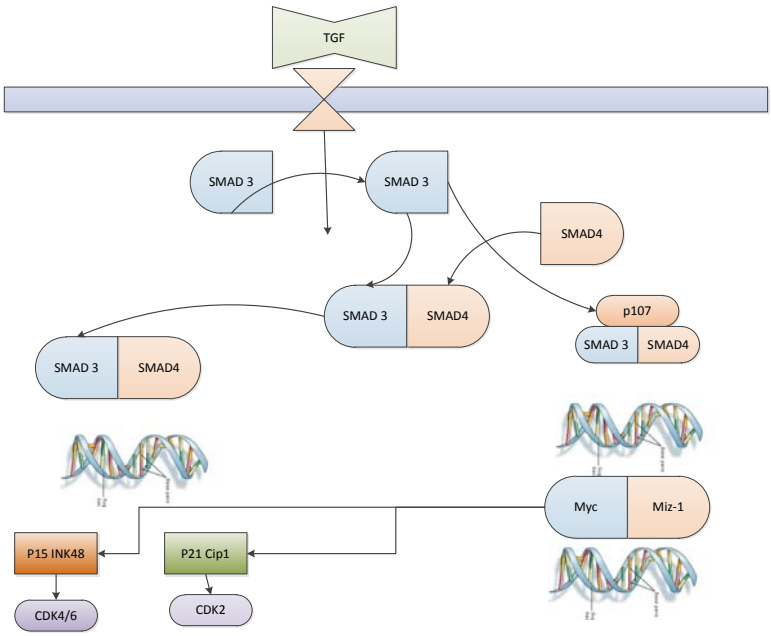
76 http://www.ncbi.nlm.nih.gov/gene/4089

77 http://pid.nci.nih.gov/search/pathway_landing.shtml?pathway_id=100160&source=BioCarta&genes_a=4089&genes_b=&what=graphic&jpg=on&ppage=1



The SMAD pathway is also detailed by NCI and one is referred to that source for further detail.

From Weinberg (p 291) we also have the SMAD4 pathway showing its immediate control of the DNA transcription.



As Weinberg states (p 292):

"… Half of all pancreatic carcinomas and more than a quarter of all colon carcinomas carry mutant inactivated Smad4 proteins. Without the presence of Smad4 neither Smad2-Smad4 nor Smad3-Smad4 complexes can form. These two complexes are the chief agents dispatched by the TGF-β receptor to the nucleus with the important assignment to shut down proliferation."

This control mechanism is shown above.

5.5.7 SPP1

SSPI is secreted phosphoprotein 1, also commonly known as Osteopontin (OPN), also known as bone sialoprotein I (BSP-1 or BNSP), early T-lymphocyte activation (ETA-1), 2ar and Rickettsia resistance (Ric), is a human gene product which is also conserved in other species[78].

From Hendig et al, they state that SPP1 is a secreted, highly acidic phosphoprotein that is involved in immune cell activation, wound healing, and bone morphogenesis and plays a major role in regulating mineralization processes in various tissues. Increased SPP1 expression is often associated with pathological calcification. Furthermore, SPP1 is a constitutive component of human skin and aorta, where it is localized to the elastic fiber and hypothesized to prevent calcification in the fibers.

SPP1 is a predominantly transcriptional regulated gene, and the *SPP1* promoter is highly conserved among different species *(22)*. Several polymorphisms in the *SPP1* gene affect SPP1 expression and have been associated with various disorders, e.g., systemic lupus erythematosus and arteriosclerosis.

SPP1 is a SIBLING glycoprotein that was first identified in osteoblasts. OPN is an important anti-apoptotic factor in many circumstances. OPN blocks the activation-induced cell death of macrophages and T cells as well as fibroblasts and endothelial cells exposed to harmful stimuli. OPN prevents non-programmed cell death in inflammatory colitis. It has been shown that OPN drives IL-17 production; OPN is overexpressed in a variety of cancers, including lung cancer, breast cancer, colorectal cancer, stomach cancer, ovarian cancer, melanoma and mesothelioma; OPN contributes both glomerulonephritis and tubulointerstitial nephritis; and OPN is found in atheromatous plaques within arteries. Thus, manipulation of plasma OPN levels may be useful in the treatment of autoimmune diseases, cancer metastasis, osteoporosis and some forms of stress. Research has implicated osteopontin in excessive scar-forming and a gel has been developed to inhibit its effect.

5.5.8 FOXO

The FOXO gene, specifically FOXO3a, forkedhead boxes zero gene, is located at 6q21 in humans and is a key nuclear transcription regulator. It has the ability to mediate cell cycle arrest,

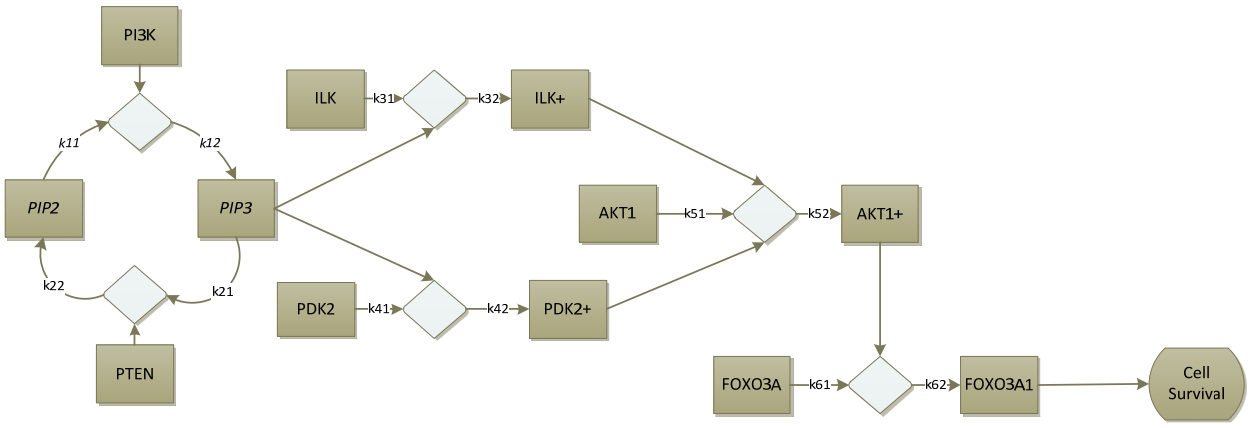
[78] Also see http://www.ncbi.nlm.nih.gov/gene/6696 also see http://www.wikigenes.org/e/gene/e/6696.html

DNA repair, and apoptosis and as such acts in many ways like a tumor suppressor gene. Loss of the FOXO gene activity may lead to uncontrolled cell growth. Also impairment or suppression of FOXO can result in impaired DNA repair capabilities as well. In a normal situation a reduced level of FOXO in a cell would lead to normal cell death however in cancerous cells this is no longer the case. As Lam et al state the FOXO molecule is key to the regulation of normal cell homeostasis. Although mutations in FOXO are not common it is the FOXO function controlled via PI3K and PTEN that often are of interest.

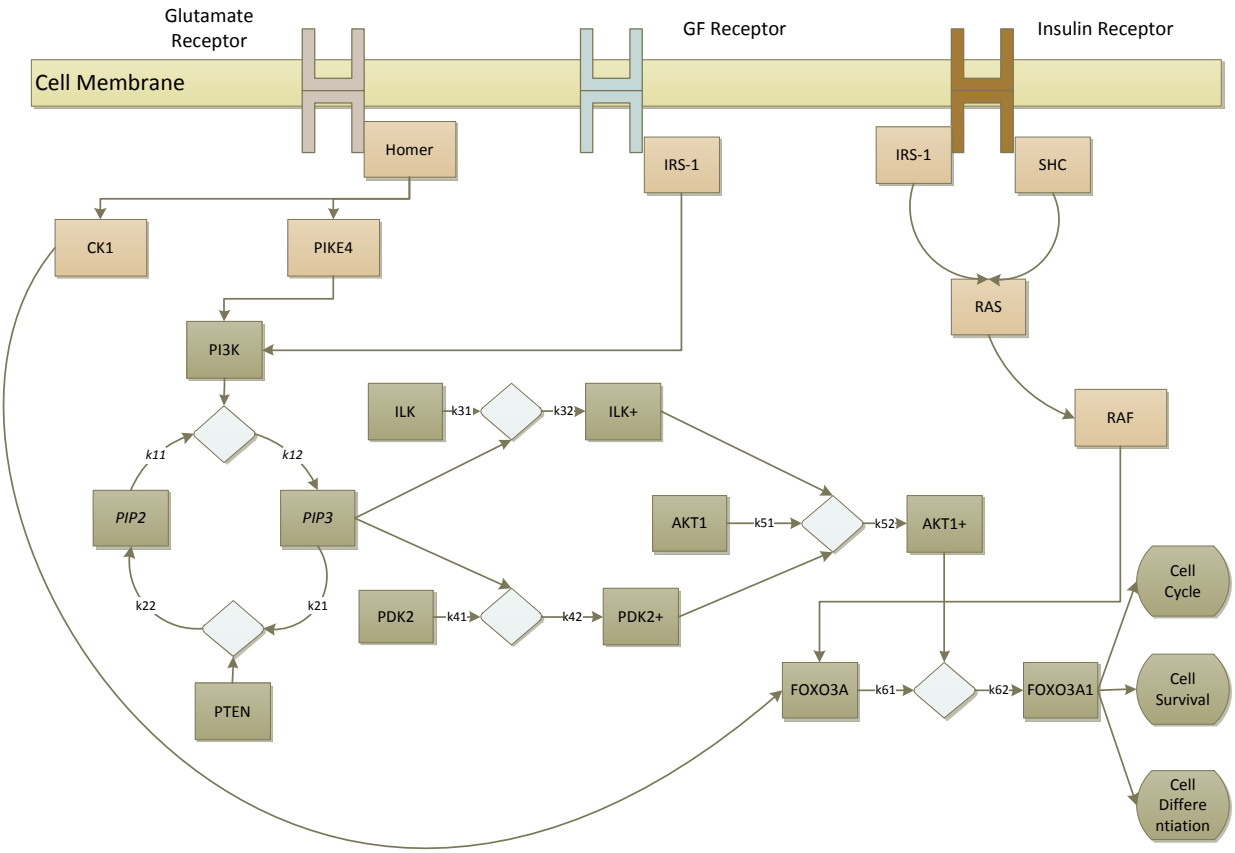
As noted by van der Heide et al, FOXO is a major player in pathways activated by Glutamate and insulin. We will depict that detail later. However the nexus to the insulin activator may also provide a connection to the role that inflammation may have in PCa and especially Type 2 Diabetes and its related hyperglycemia.

FOXO is a key element in the PI3K pathway and has its control facilitated by such elements as PTEN, growth factors, insulin and glutamate. As Essaghir et al state, in the absence of growth factors, FOXO remains in the nucleus and FOXO up-regulates genes which inhibit cell cycle such as p27 KIP1 and p21 WAF1. It also promotes apoptosis via the Fas ligand, Bim and TRAIL, and decreases oxidative stress. As a blocker of cell growth therefore FOXO is often considered as a tumor suppressor. There has been a recent interest in dealing with the FOXO gene directly as a way to control certain cancers as discussed by Yang et al (2010).

One view of the FOXO pathway is shown as follows:



However we can also add the receptors which are drivers of the internal elements. We do that as follows. This shows the multiple ligan responses, with limited detail regarding reactions. We have taken the pathway we have analyzed elsewhere and included it as a core element of the FOXO control mechanism.



FOXO is a facilitator gene, it facilitates homeostasis of the cell. However it is regulated by many genes above it which are often inhibited in their normal functions in a cancer cell.

As Lam et al state:

The PI3K signal transduction pathway critically regulates cell proliferation, differentiation and apoptosis. Perturbation in the PI3K signalling pathway is strongly implicated in the pathogenesis of many diseases, including heart and neural diseases, autoimmune/inflammatory disorders, cancer and the development of chemo- and endocrine-resistance in tumor cells.

Constitutive activation of the PI3K pathway, a hallmark of many cancers, is commonly a consequence of enhanced expression of genes that encode either class I PI3K subunits or PKB (protein kinase B) or is a result of genetic mutations that inhibit negative regulators of the pathway. For example, somatic deletions or mutations of PTEN (phosphatase and tensin homologue deleted on chromosome 10), an antagonist of the PI3K pathway, have been identified in a large proportion (12–60%) of human tumours of different tissue origins.

They continue:

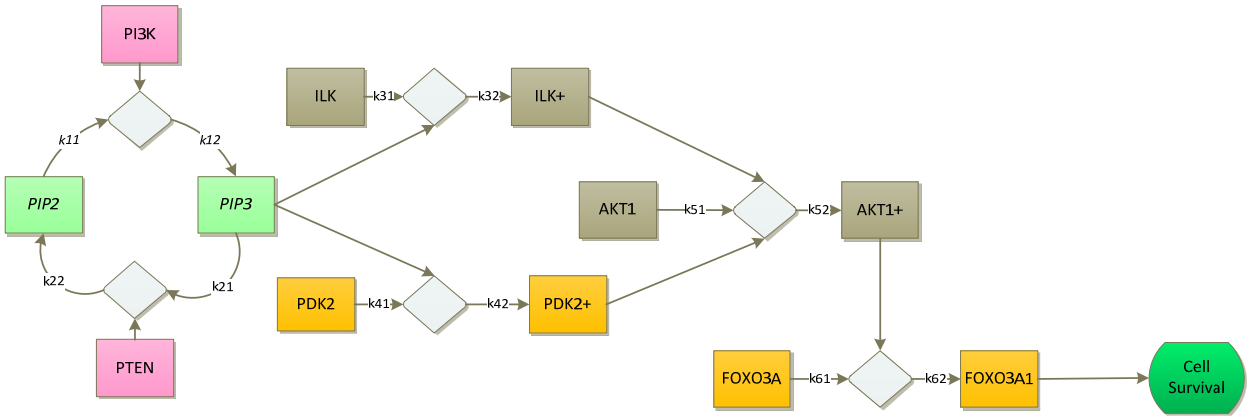
In mammals, the ability of FOXO factors to mediate cell-cycle arrest, DNA repair and apoptosis makes them attractive candidates as tumor suppressors. Loss of FOXO function can lead to uncontrolled cell proliferation. Furthermore, reduced ability to repair damaged DNA due to

impaired FOXO activity may also result in genomic instability and carcinogenesis. Finally, a deficiency in FOXO proteins in abnormal and damaged cells that would normally undergo programmed cell death may result in tumor development and expansion.

FOXO transcription factors control cell proliferation and survival by regulating the expression of genes involved in cell-cycle progression [e.g. $p27_{Kip1}$, p130(RB2), cyclin D1/2 and Bcl-6 (B-cell lymphocytic leukemia proto-oncogene 6)] and apoptosis [e.g. Bim, Fas ligand, TRAIL (tumor-necrosis-factor-related apoptosis inducing ligand) and Bcl-X_L. Thus one way by which PKB and the related SGK promote cell survival is by phosphorylating FOXOs, which results in their sequestration in the cytoplasm away from cell death-inducing genes. PKB phosphorylation also reduces the DNA-binding ability of FOXO and enhances its degradation.

Common FOXO target genes that mediate apoptosis include bNIP3 and BCL2L11, which encode the pro-apoptotic Bcl-2 family members, bNIP3 and Bim. Furthermore, FOXOs also indirectly down-regulate the expression of the pro-survival Bcl-2 family member Bcl-X_L by inducing the expression of the transcriptional repressor Bcl-6 . In neurons, FOXO3a triggers cell death circuitously by inducing the expression of Fas Ligand, which triggers programmed cell death through the death receptor pathway.

Thus FOXO control is a strategic part of controlling cell growth and stability.

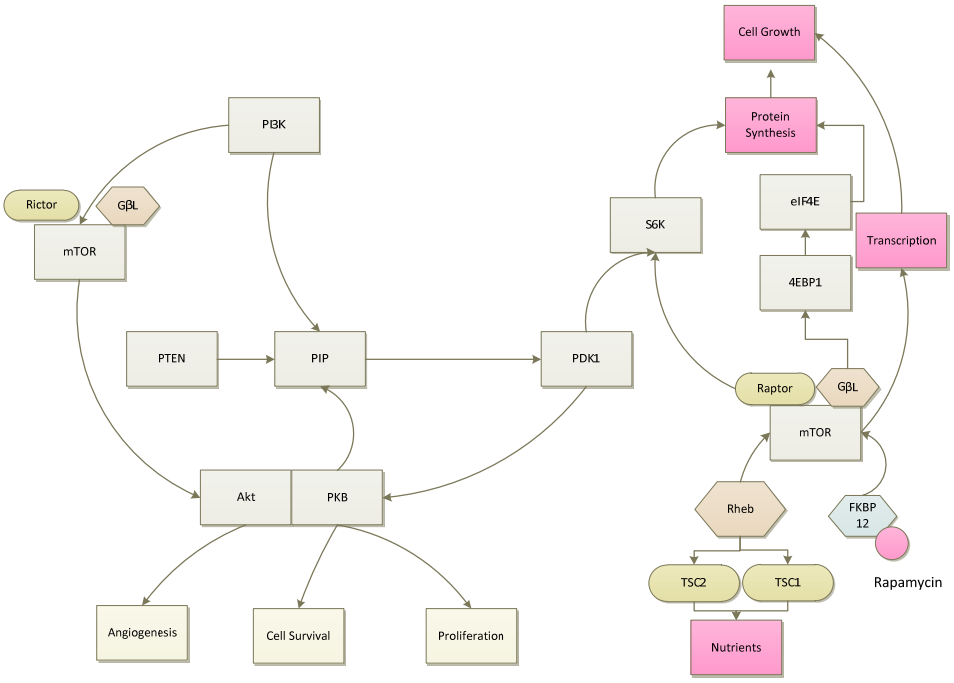


It should also be noted that we have indicated transition rate or reaction rates in the above. This as we shall demonstrate later is a "gross" reaction assumption because we have a small and countable number of proteins, not a density upon which these rates are typically proffered. We shall examine this in detail later.

5.5.9 mTOR

mTOR, the mammalian target of rapamycin, is a gene product (1p36.2) is a protein which acts in a critical manner in interconnecting the genetic circuits in mammals, and especially man. It fundamentally controls glucose transport and protein synthesis. The pathway depicted below is a modification of the graphic from Weinberg (p 785) which shows mTOR in its two modes, one with Raptor assisting and one with Rictor. The Rictor/mTOR mode activates the Akt pathway

via the placement of a phosphate and the manages the protein synthesis portion. The inclusion of rapamycin will block the Raptor/mTOR path and reduce the protein synthesis and cell growth portion. The inhibitory effect on Akt/PKB by rapamycin is assumed to be the main factor in its anti-cancer effects.



Looking at the complexity of the mTOR pathway it presents an interesting one for addressing PCa. Kinkaide et al (2008) indicate:

Among the major signaling networks that have been implicated in advanced prostate cancer are the AKT/mammalian target of rapamycin (AKT/mTOR) and MAPK pathways. Indeed, deregulated expression and/or mutations of the phosphate and tensin homolog tumor suppressor gene (PTEN) occur with high frequency in prostate cancer, leading to aberrant activation of AKT kinase activity as well as its downstream effectors, including the mTOR signaling pathway. In addition, many prostate tumors display deregulated growth factor signaling, which may result in activation of MAPK kinase 1 (MEK) kinase and ultimately ERK MAP.

Notably, previous studies have demonstrated that the AKT/mTOR and MAPK signaling pathways are alternatively and/ or coordinately expressed in advanced prostate cancer and function cooperatively to promote tumor growth and the emergence of hormone- refractory disease. These observations formed the basis for our hypothesis that targeting these signaling pathways combinatorially may be effective for inhibiting tumorigenicity and androgen independence in prostate cancer.

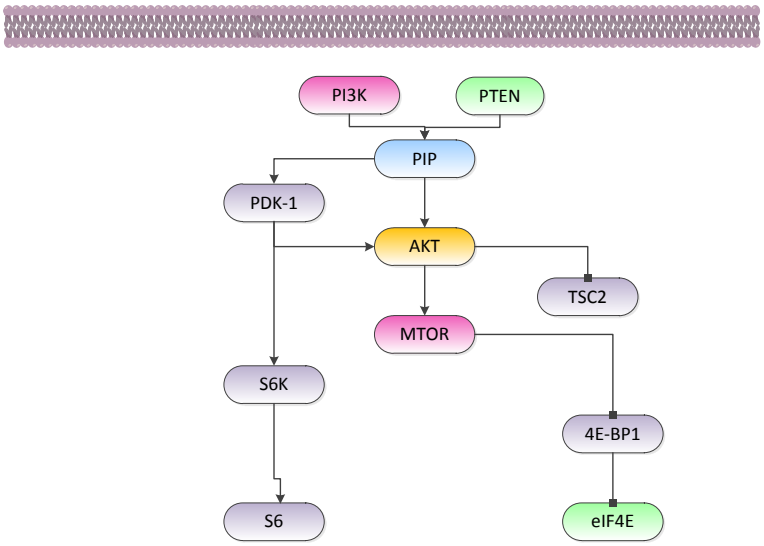
Kinkaide et al also demonstrate the creation of HGPIN via their work. This represents another pathway of HGPIN to PCa.

LoPiccolo et al state:

The PI3K/Akt/mTOR pathway is a prototypic survival pathway that is constitutively activated in many types of cancer. Mechanisms for pathway activation include loss of tumor suppressor PTEN function, amplification or mutation of PI3K, amplification or mutation of Akt, activation of growth factor receptors, and exposure to carcinogens. Once activated, signaling through Akt can be propagated to a diverse array of substrates, including mTOR, a key regulator of protein translation. This pathway is an attractive therapeutic target in cancer because it serves as a convergence point for many growth stimuli, and through its downstream substrates, controls cellular processes that contribute to the initiation and maintenance of cancer.

Moreover, activation of the Akt/mTOR pathway confers resistance to many types of cancer therapy, and is a poor prognostic factor for many types of cancers. This review will provide an update on the clinical progress of various agents that target the pathway, such as the Akt inhibitors perifosine and PX-866 and mTOR inhibitors (rapamycin, CCI-779, RAD-001) and discuss strategies to combine these pathway inhibitors with conventional chemotherapy, radiotherapy, as well as newer targeted agents. We (show) how the complex regulation of the PI3K/Akt/mTOR pathway poses practical issues concerning the design of clinical trials, potential toxicities and criteria for patient selection.

LoPiccolo et al show the more simplified pathway as follows:

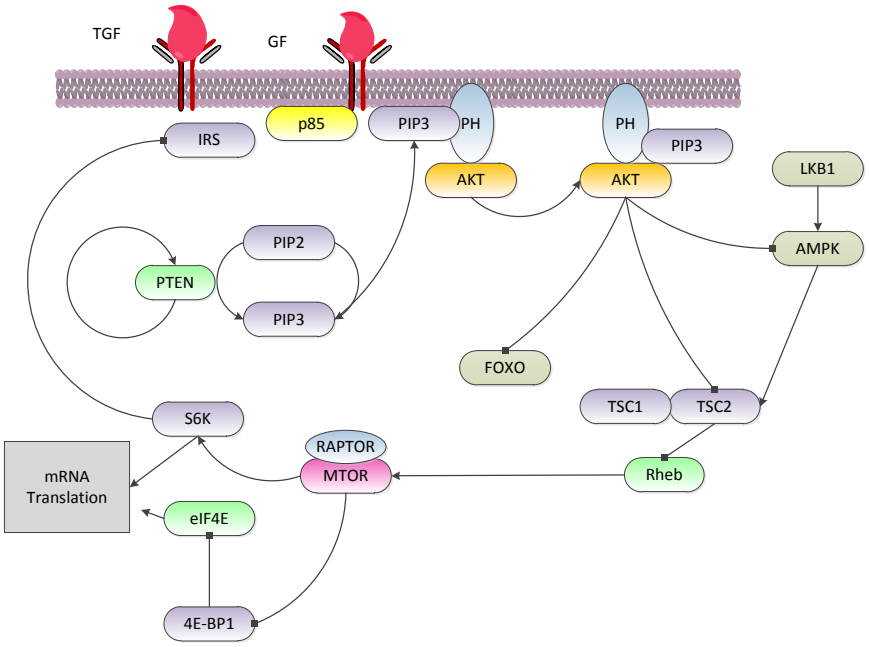


As we have shown with the more complex Weinberg model, here mTOR and PTEN play a strong role in the overall control. The authors show the points of possible control. The complexity of the pathways will be a challenge. It is less an issue of size complexity than a feedback and instability complexity. E Nelson et al (2007) have demonstrated similar results as well.

Other researchers have also posited other simple models. We demonstrated the one by Hay as has been stated:

The downstream effector of PI3K, Akt, is frequently hyperactivated in human cancers. A critical downstream effector of Akt, which contributes to tumorigenesis, is mTOR. In the PI3K/Akt/mTOR pathway, Akt is flanked by two tumor suppressors: PTEN, acting as a brake upstream of Akt, and TSC1/TSC2 heterodimer, acting as a brake downstream of Akt and upstream of mTOR.

In the absence of the TSC1/TSC2 brake, mTOR activity is unleashed to inhibit Akt via an inhibitory feedback mechanism. Two recent studies used mouse genetics to assess the roles of PTEN and TSC2 in cancer, underscoring the importance of Akt mTOR interplay for cancer progression and therapy.



The Baldo et al model is quite similar to the Weinberg model shown initially. It clearly demonstrates the overall controlling influence of mTOR. As Baldo et al state:

There is a great body of evidence supporting consideration of the mTOR signaling system as an important network in cell regulation, differentiation and survival . mTOR is a sensor of mitogen, energy and nutritional levels, acting as a "switch" for cell-cycle progression from phase G1 to phase S.

The antibiotic Rapamycin, a potent mTOR inhibitor, has been known to the National Cancer Institute and recognized for its potential anticancer properties since the 1970s. The observation that cell lines from different cancer types exposed to low doses of Rapamycin underwent cell-

cycle arrest in phase G1, provided the basis for considering mTOR as a target for cancer therapy.

Development of mTOR inhibitor compounds has proceeded empirically due to the lack of understanding of the precise molecular targets and the required dose of the new compounds . The development of Rapamycin analogs ("Rapalogs"), but also of other, structurally different, mTOR inhibitors, was directed at the selection of specific cancer type sensitivity and an optimization of pharmaceutical forms.

To give an example, Temsirolimus revealed clinical responses in patients with renal cell carcinoma in advanced stage. Temsirolimus was approved by the FDA on May 2007 for this therapeutic use and is being investigated in clinical trials for other cancer types (breast cancer, lymphoma, renal cancer, glioblastoma); significantly there are a considerable number of clinical studies involving mTOR inhibitors currently active worldwide…

The mTOR pathway controls cell size and cellular proliferation*.…nutrient metabolism, mRNA translation and cell survival control. Disruption of TOR leads to early embryonic death in flies and mammalian cells, indicating mTOR plays an important role in regulating cell survival. … deregulation of several mTOR components leads to modified cell proliferation patterns and, on the other, that many mTOR components are deregulated in several human cancers.*

*… **Therefore, inhibition of mTOR leads to slowing or arrest of cells in the G1 phase**. Translational control may have an important role in the balance of cell survival and death, and hence for apoptosis. Importantly, components of mTOR are deregulated in some human cancers, for example, breast and colon. Alteration of PI3-K/Akt is frequently observed in head and neck cancer .*

PTEN, a phosphatase that acts on PIP3 to convert it to PIP2, normally regulates the mTOR pathway negatively, and shows decreased activity in some tumors. A strong relation seems to exist between the sensitivity to the effect of Rapamycin and PTEN loss or deregulation. PTEN is frequently mutated in several cancers and in cancer-like syndromes like Cowden and Proteus syndromes…

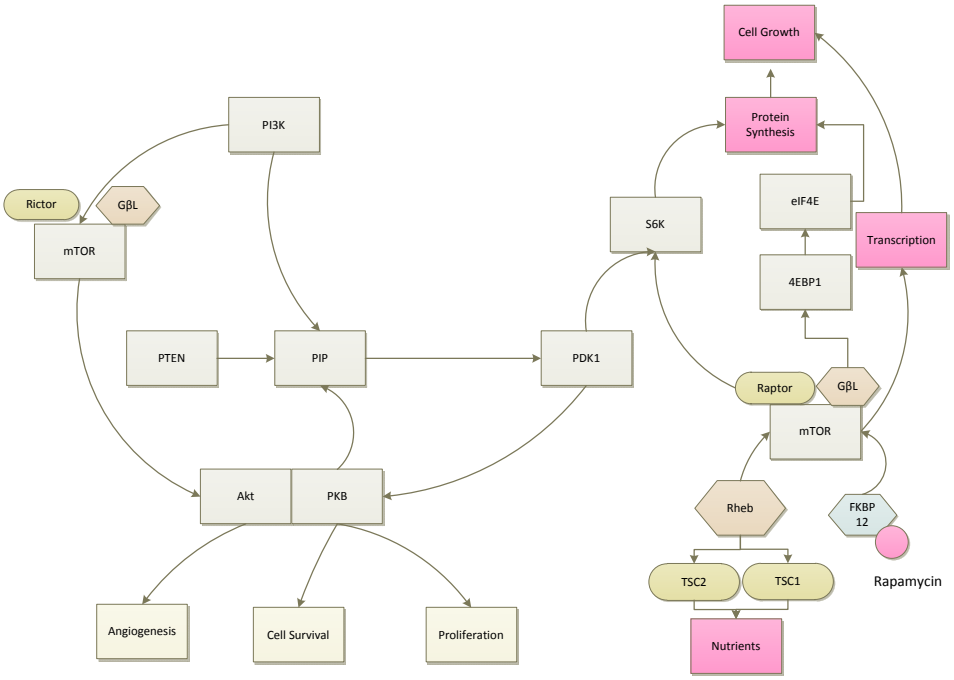
Loss of PTEN function can occur in 26-80% of endometrial carcinomas, …recent studies of human prostate cancer have shown that loss of PTEN is strongly associated with more aggressive cancers. The relationship between PTEN status and sensitivity to rapalogs has been questioned by several investigators. Some attention has recently been dedicated to the role of the mTORC2 complex in the mTOR pathway.

In fact this complex, believed until recently to be completely insensitive to the effect of Rapamycin, after long-term exposure to Rapamycin is able to prevent mTOR-mediated Akt phosphorylation and the activation of the mTOR pathway. Another component, the TSC1/TSC2 complex located upstream of mTOR, is predicted to integrate signals derived from nutrients, cellular energy status and hypoxia into a common growth regulatory signal to the mTORC1 complex.

As Easton and Houghton state:

Proteins regulating the mammalian target of rapamycin (mTOR), as well as some of the targets of the mTOR kinase, are overexpressed or mutated in cancer. Rapamycin, the naturally occurring inhibitor of mTOR, along with a number of recently developed rapamycin analogs (rapalogs) consisting of synthetically derived compounds containing minor chemical modifications to the parent structure, inhibit the growth of cell lines derived from multiple tumor types in vitro, and tumor models in vivo.

Results from clinical trials indicate that the rapalogs may be useful for the treatment of subsets of certain types of cancer. The sporadic responses from the initial clinical trials, based on the hypothesis of general translation inhibition of cancer cells are now beginning to be understood owing to a more complete understanding of the dynamics of mTOR regulation and the function of mTOR in the tumor microenvironment. This review will summarize the preclinical and clinical data and recent discoveries of the function of mTOR in cancer and growth regulation.



The other observation here is that we often find multiple characterizations of the pathways. Namely there is no canonical form, and often a pathway is depicted to demonstrate a specific protein function. Thus we may see an emphasis on one set of proteins while others are neglected. As much as we currently attempt to unify this process we are left somewhat adrift in model development at this stage. This can be exemplified by now looking at the next section on LKB1. There we show its control over PTEN whereas in an earlier model we have it controlling AMPK. In reality there are multiple links as we have discussed. The literature can be even more confusing on this issue as well.

5.5.10 LKB1

LKB1 has been demonstrated to be the underlying control element in Peutz-Jeghers syndrome, a proliferative melanocytic genetically dominant disorder. It controls certain pathways and as a result can be considered as a candidate in the development and progression of melanoma. Generally LKB1 is a gene whose protein stabilizes the growth and location of melanocytes. Understanding its impact in Peutz-Jeghers allows one to examine what happens when its function is suppressed in melanoma. Albeit not an initiator in the process, its aberration in a melanocyte argues for movement and loss of control.

In a recent paper by Liu et al the authors examine this premise and conclude that loss of LKB1 is significant especially in metastatic evolution. As Liu et al state:

Germline mutations in LKB1 (STK11) are associated with the Peutz-Jeghers syndrome (PJS), which includes aberrant mucocutaneous pigmentation, and somatic LKB1 mutations occur in 10% of cutaneous melanoma. By somatically inactivating Lkb1 with K-Ras activation (±p53 loss) in murine melanocytes, we observed variably pigmented and highly metastatic melanoma with 100% penetrance. LKB1 deficiency resulted in increased phosphorylation of the SRC family kinase (SFK) YES, increased expression of WNT target genes, and expansion of a $CD24^+$ cell population, which showed increased metastatic behavior in vitro and in vivo relative to isogenic $CD24^-$ cells. ***These results suggest that LKB1 inactivation in the context of RAS activation facilitates metastasis by inducing an SFK-dependent expansion of a prometastatic, $CD24^+$ tumor subpopulation.***

Earlier work by Zheng et al noted:

The LKB1-AMPK signaling pathway serves as a critical cellular sensor coupling energy homeostasis to cell growth, proliferation, and survival. However, how tumor cells suppress this signaling pathway to gain growth advantage under conditions of energy stress is largely unknown.

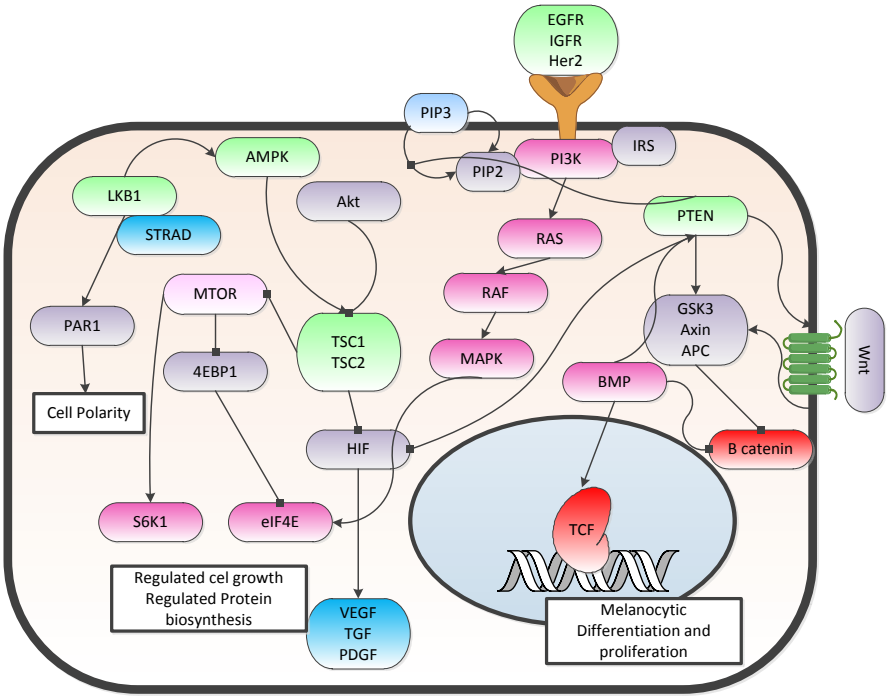
Here, we show that AMPK activation is suppressed in melanoma cells with the B-RAF V600E mutation and that downregulation of B-RAF signaling activates AMPK. We find that in these cells LKB1 is phosphorylated by ERK and Rsk, two kinases downstream of B-RAF, and that this phosphorylation compromises the ability of LKB1 to bind and activate AMPK. Furthermore, expression of a phosphorylation-deficient mutant of LKB1 allows activation of AMPK and inhibits melanoma cell proliferation and anchorage-independent cell growth.

Our findings provide a molecular linkage between the LKB1-AMPK and the RAF-MEK-ERK pathways and suggest that suppression of LKB1 function by B-RAF V600E plays an important role in B-RAF V600E-driven tumorigenesis.

Thus Zheng et al putatively identified these two pathways as sources for melanoma development. Liu et al appear to have extended this to metastasis.

Now in a paper by Bauer and Stratakis the authors provide an excellent overview of the controlling pathways. We provide a revised version of their pathway controls in a normal

melanocyte below. This provides a description of the normal homeostatic pathways within a melanocyte.

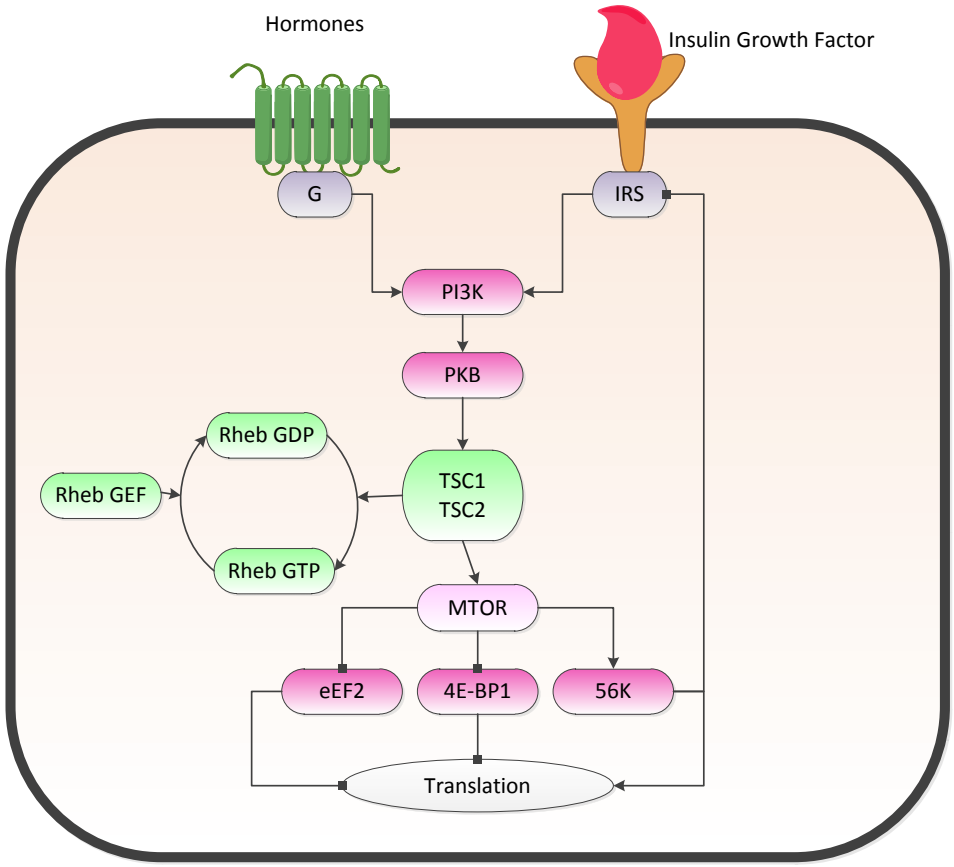


From: Bauer and Stratakis

The LKB1 gene, also called STK11, which encodes a member of the serine/threonine kinase, regulates cell polarity and functions as a tumour suppressor. This is clearly demonstrated in the above. Now recall that mTOR is a protein kinase and is a key regulator of cell growth[79]. mTOR stimulates mRNA translation thus facilitating the conversion into proteins. mTOR also facilitates the formation of ribosomes which as an important condition of cell growth under specific physiological conditions. Through the effects of mTOR on the ribosome machinery it becomes a significant factor in increasing translational activity in a cell.

We demonstrate graphically how mTOR function in some detail below:

[79] See Marks et al pp 335-345.



As Marks et al state regarding the above flow we have (p 337):

Activation and effects of the mTOR protein kinase *By inactivating the GAP TSC2 of the small G-protein Rheb, extracellular signals stimulating the PI3K-PKB signaling cascade prompt Rheb to activate mTOR. mTOR enhances the activity of the protein kinase S6K and represses 4E-BP1 and eEF2 activities, resulting in an increased rate of translation (whether 4E-BP1 and eEF2 kinase are phosphorylated directly by mTOR, as shown here, or by S6K or by both kinases is not entirely clear).*

mTOR may also be directly phosphorylated and activated by PKB.

A stimulatory effect resembling that of PKB has the MAP kinase ERK connecting mTOR signaling with mitogenesis (not shown). mTOR is also activated by nutrients such as amino acids and sugars along an ill-defined pathway that seems to include a class III PI3K.

The red dotted line (we use squared ends as compared to arrow ends) shows the negative feedback of insulin signaling: S6K phosphorylates and inactivates the insulin-specific docking protein IRS. This effect is augmented by overnutrition (leading to increased insulin release) and provides one of the causes of diabetes. Also shown is the activation of the Rheb-GAP TSC2 by 5′-AMP-dependent protein kinase (AMPK) that results in an inhibition of mTOR signaling and protein synthesis and protects the cell in situations of energy deficiency.

Now Liu et al state regarding this pathway model:

Two independent pathways appear to be critically important in regulating cell growth in response to nutrient supply and mitogenic stimulation:

(i) the PKA/PRKAR1A-LKB1 tumour suppressor protein pathway, acting via AMPK, and

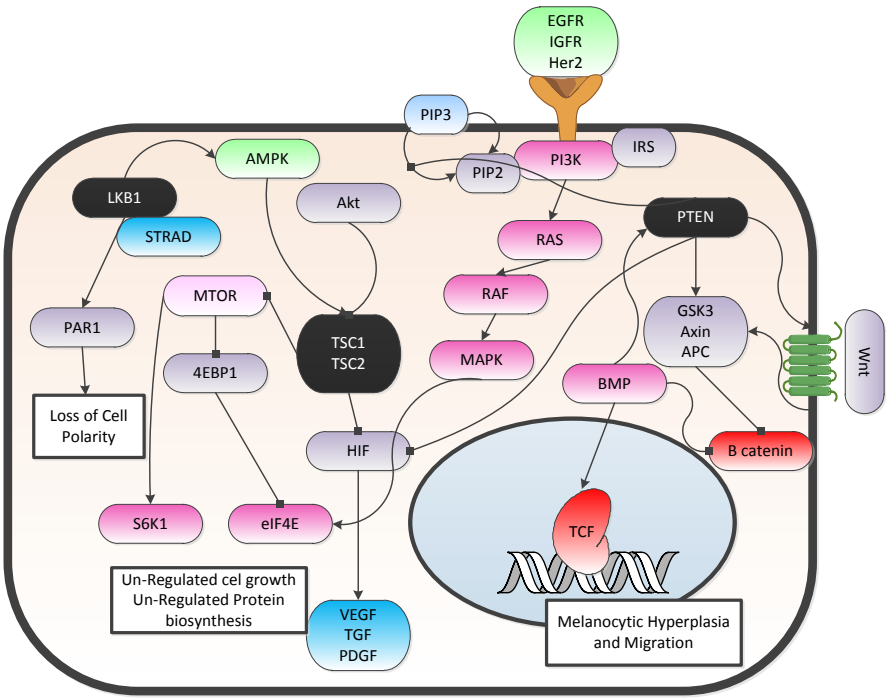
(ii) the PI3K/AKT pathway.

Recent evidence suggests that the tumour suppressor gene complex, TSC1/TSC2, orchestrates the signal from both pathways to the downstream target, mTOR, which in turn regulates the ribosomal protein S6 and 4EBP-1, a repressor of the translational initiation factor eIF4E. In this model, at times of nutrient stress LKB1/AMPK activation of the TSC1/TSC2 complex results in inhibition of mTOR and a decrease in protein synthesis.

Under stimulation of mitogenic pathways, PI3K phosphorylates PIP2 to PIP3 resulting in recruitment of AKT to the membrane where it is activated by PDK1. Activated AKT inhibits the TSC1/TSC2 tumour suppressor complex leading to increased mTOR activity. In the later pathway, PTEN antagonises PIP3 action through dephosphorylation, and thus provides an ''off'' switch for regulating mitogenic pathway induced cellular growth and proliferation.

Cross talk of several other pathways appears to play important regulatory roles in the lentiginoses syndromes to include the Ras/MAPK pathway in the regulation of translation, the LKB1 pathway in cellular polarity, the AKT pathway (as well as the TSC1/TSC2 complex) in the regulation of the Wnt/GSK3b/b-Cat pathway, and the BMP pathway in the regulation of PTEN (see text for further discussion). Lastly, both PTEN and mTOR appear to have negative regulatory effects on VEGF through loss of stabilisation of the hypoxia inducible transcription factor 1 (HIF1).

When LKB1 is inactivated we have the following changes observed in a melanocyte. Note the deactivation of normal LKB1 proteins as well as a PTEN loss of function. We then have:



From: Bauer and Stratakis

These models or Bauer and Stratakis are compelling and establish a paradigm which the work of Liu et al can be considered.

Let us go back to LKB1 and its function. From NLM database we have[80]:

LKB1 is a primary upstream kinase of adenine monophosphate-activated protein kinase (AMPK), a necessary element in cell metabolism that is required for maintaining energy homeostasis. It is now clear that LKB1 exerts its growth suppressing effects by activating a group of other ~14 kinases, comprising AMPK and AMPK-related kinases.

Activation of AMPK by LKB1 suppresses growth and proliferation when energy and nutrient levels are scarce. Activation of AMPK-related kinases by LKB1 plays vital roles maintaining cell polarity thereby inhibiting inappropriate expansion of tumour cells. A picture from current research is emerging that loss of LKB1 leads to disorganization of cell polarity and facilitates tumour growth under energetically unfavorable conditions. Also it is known as PJS; LKB1; hLKB1.

This gene, which encodes a member of the serine/threonine kinase family, regulates cell polarity and functions as a tumor suppressor. Mutations in this gene have been associated with Peutz-Jeghers syndrome, an autosomal dominant disorder characterized by the growth of polyps in the gastrointestinal tract, pigmented macules on the skin and mouth, and other neoplasms. Alternate

[80] http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene&cmd=retrieve&dopt=default&rn=1&list_uids=6794

transcriptional splice variants of this gene have been observed but have not been thoroughly characterized.

From the results of Shaw et al we have[81]:

AMP-activated protein kinase (AMPK) is a highly conserved sensor of cellular energy status found in all eukaryotic cells. AMPK is activated by stimuli that increase the cellular AMP/ATP ratio. Essential to activation of AMPK is its phosphorylation at Thr-172 by an upstream kinase, AMPKK, whose identity in mammalian cells has remained elusive.

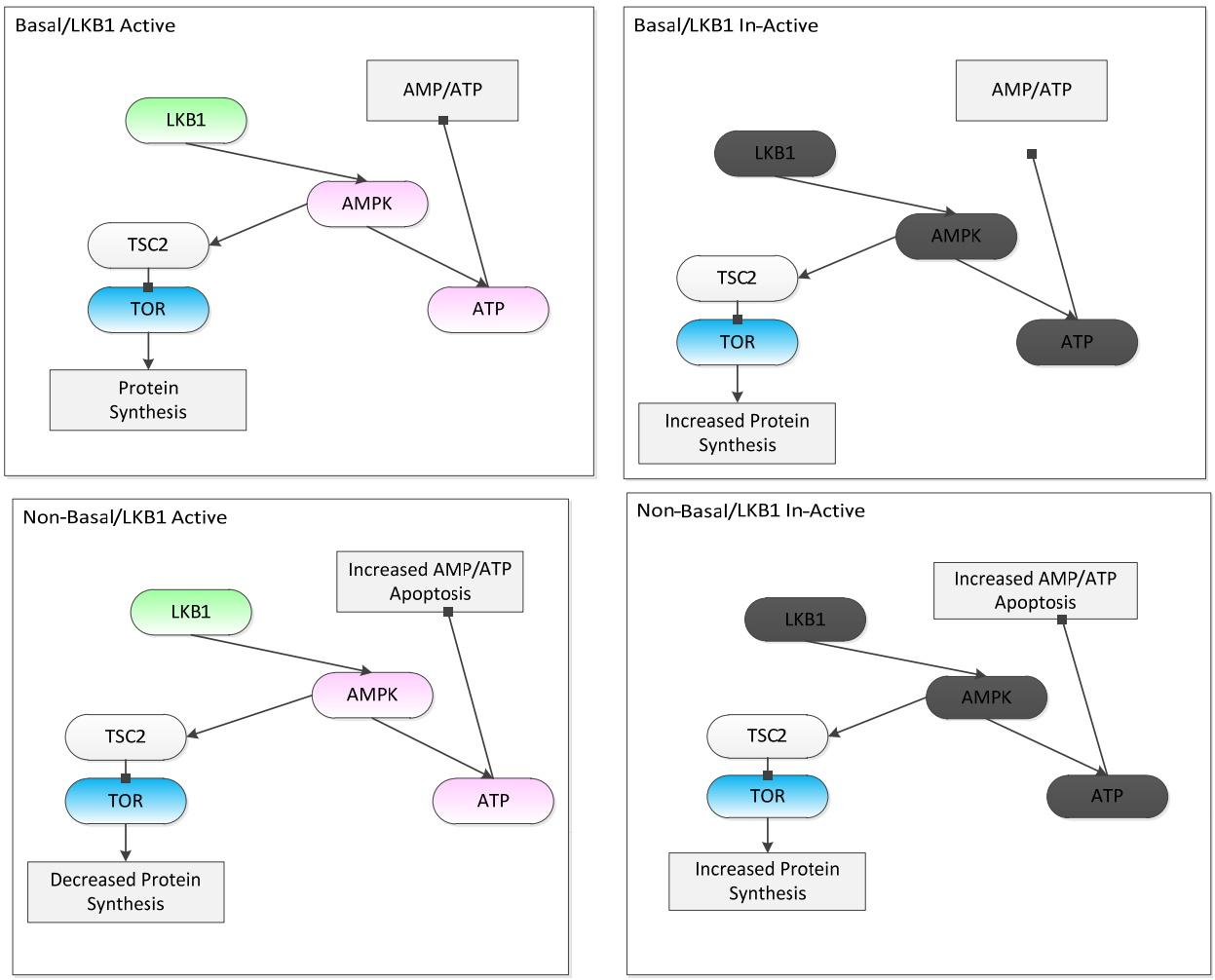
Here we present biochemical and genetic evidence indicating that the LKB1 serine/threonine kinase, the gene inactivated in the Peutz-Jeghers familial cancer syndrome, is the dominant regulator of AMPK activation in several mammalian cell types. We show that LKB1 directly phosphorylates Thr-172 of AMPKalpha in vitro and activates its kinase activity.

LKB1-deficient murine embryonic fibroblasts show nearly complete loss of Thr-172 phosphorylation and downstream AMPK signaling in response to a variety of stimuli that activate AMPK. Reintroduction of WT, but not kinase-dead, LKB1 into these cells restores AMPK activity. Furthermore, we show that LKB1 plays a biologically significant role in this pathway, because LKB1-deficient cells are hypersensitive to apoptosis induced by energy stress.

On the basis of these results, we propose a model to explain the apparent paradox that LKB1 is a tumor suppressor, yet cells lacking LKB1 are resistant to cell transformation by conventional oncogenes and are sensitive to killing in response to agents that elevate AMP. The role of LKB1/AMPK in the survival of a subset of genetically defined tumor cells may provide opportunities for cancer therapeutics.

Also Shaw et al demonstrate several ways in which LKB1 can function when activated in vivo from either a basal or non-basal state. The description can be shown in the following Figure:

[81] http://www.ncbi.nlm.nih.gov/pubmed/14985505



Shaw et al describe the above as follows:

Model for LKB1 as a sensor of low energy and negative regulator of tumorigenesis and apoptosis. Under basal conditions, LKB1 serves as a sensor of low energy, keeping ATP-consuming processes including protein synthesis in check via AMPK phosphorylation of TSC2.

In response to stresses such as low glucose, hypoxia, nutrient deprivation, or mitochondrial poisons, LKB1 phosphorylates AMPK, which shuts off ATP-consuming processes and up-regulates ATP production to offset the elevated AMP/ATP ratio. This activity prevents the cells from going into apoptosis in response to elevated AMP. In LKB1-deficient cells, under some basal conditions, there may be increases in TOR signaling due to the lack of TSC2 phosphorylation by AMPK, resulting in increased growth or tumorigenic potential. In response to further increases in intracellular AMP, these cells have no mechanism to offset the elevated AMP and go straight into apoptosis.

However, although this is an interesting and compelling description of the metastatic driving factors, there are a multiple set of issues still outstanding:

1. Metastatic behavior implies the ability of the malignant melanocyte to migrate at will within the body. Movement of the melanocyte requires breaking of the E cadherin bonds with the adjacent keratinocytes. Thus is there a sequence of genetic changes and how does this putative mechanism relate to that of the E cadherin mechanism.

As Baas et al state:

A second prominent aspect of polarized simple epithelia is the presence of junctional complexes at the apical boundaries between neighboring cells. These junctions form an impenetrable seal between cells and provide strength to the epithelial sheet by serving as anchoring sites for cytoskeletal elements including the brush border.

We found that LS174T cells do not express junctional proteins, such as ZO-1, and are homozygous mutant for E-cadherin. By contrast, DLD-1 cells are capable of forming tight junctions and adhesion junctions when grown to confluency and appear to express most junctional components already at low-cell density.

We determined the localization of the tight junction component ZO-1 and of the adherens junction protein p120 before and after activation of LKB1 in DLD-1-W5 cells grown at very low density.

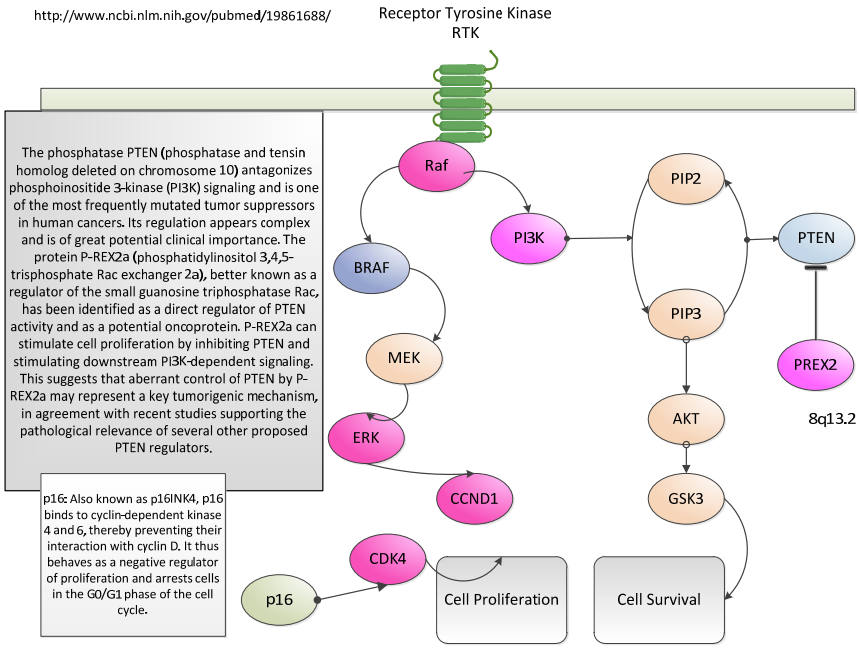
2. LKB1 is a gene related to the control from decreased nutrients. However we have the angiogenesis issue related to the increased nutrition of malignant cells. However on the counter side we have the Warburg effect as a counter to normal metabolism, namely cancer cells are anaerobic metabolic systems. What is the balance between the two?

3. Is the LKB1 mutation one of random gene mutations or is it a direct consequence of other downstream mutations? Is perhaps this loss of LKB1 a result of some induced miRNA effect in vivo?

The following is a list and description of the key genes/proteins seen in this specific set of reactions.

5.5.11 PREX2

PREX2 controls PTEN and it was observed that mutations there inhibited PTEN. Berger et al have published a set of results linking PREX changes to melanoma. PREX can as shown below turn off PTEN which then controls a set of other pathway elements that will result in loss of homeostatic control.



As Hayden states in a summary of Berger et al:

Berger and his colleagues also found potentially damaging PREX2 mutations in 14% of 107 tumours that were not part of the initial study. And when they transplanted human skin cells containing PREX2 mutations into mice that had been engineered to develop skin cancer, four of the six different PREX2 mutations accelerated development of the tumours in mice. This led the researchers to suggest that PREX2 might have a similar role in human skin cancers.

There is always the risk in murine models that the pathways may be different, controlled by factors such as other ligands and having other variable intercellular dynamics. This has been, it can be argued, some of the difficulty in the Goldstein model for PCa.

PREX2 itself is probably not a good drug target, because the mutations found in the gene do not cluster in any single location that might be easily pinpointed by a drug, says cancer researcher Levi Garraway, also at the Broad Institute, who led the study. However, Garraway says, the discovery should help researchers to improve their knowledge of the biological pathways that are disrupted in melanomas. In turn, that could lead scientists to genes and proteins in other parts of those pathways that might be better drug targets.

The pathway issue keeps coming back as a dominant factor. We show BRAF and PTEN above and BRAF is now a partially controllable mutation. Broadly speaking kinase inhibitors are now somewhat well understood. PREX2 however does not fall in that category.

PREX2 also seems to work differently from BRAF and NRAS, which are considered to be 'classic' oncogenes — overactive genes that have the potential to cause cancer and which are

often mutated in the same ways. By contrast, the various PREX2 mutations identified by Berger and his colleagues occurred in different places in the protein. All seemed to lead the cell to make more of the protein than usual, rather than making the protein itself overactive.

One of the issues which seem to be coming to the fore in pathways is the details of the pathway dynamics or kinetics. This is an example of a yet to be determined kinetic model.

The summary of the article states:

Melanoma is notable for its metastatic propensity, lethality in the advanced setting and association with ultraviolet exposure early in life. To obtain a comprehensive genomic view of melanoma in humans, we sequenced the genomes of 25 metastatic melanomas and matched germline DNA. A wide range of point mutation rates was observed: lowest in melanomas whose primaries arose on nonultraviolet-exposed hairless skin of the extremities (3 and 14 per megabase (Mb) of genome), intermediate in those originating from hair-bearing skin of the trunk (5–55 per Mb), and highest in a patient with a documented history of chronic sun exposure (111 per Mb).

Analysis of whole-genome sequence data identified PREX2 (phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor 2)—a PTEN-interacting protein and negative regulator of PTEN in breast cancer2—as a significantly mutated gene with a mutation frequency of approximately 14%in an independent extension cohort of 107 human melanomas. PREX2 mutations are biologically relevant, as ectopic expression of mutant PREX2 accelerated tumour formation of immortalized human melanocytes in vivo. Thus, whole-genome sequencing of human melanoma tumours revealed genomic evidence of ultraviolet pathogenesis and discovered a new recurrently mutated gene in melanoma.

Now the PTEN control element is key in many cancers, such as prostate and many others.

As Fine et al state in their discussion of PREX2 and its effect on PTEN:

The P-REX2a gene is located on chromosome 8q13, a region of frequent amplification in breast, prostate, and colorectal cancers which has also been linked to aggressive cancer phenotypes and metastatic progression. We investigated P-REX2a expression by qRTPCR in a breast tumor data set thoroughly annotated for PI3K pathway alterations. P-REX2a showed a significant two-tailed association with PTEN status (p=0.027) and the median PREX2a expression was 3 fold greater in tumors that retained PTEN than in those that did not.

Additionally, gene expression data sets from other cancer databases demonstrate increased expression of P-REX2a in various tumors including breast and prostate compared to that in normal tissues. Mutations in P-REX2a were not found in a breast tumor mutation survey, however, our analysis of publicly available databases yielded numerous somatic mutations in P-REX2a in other tumors including those of the colon, pancreas and lung, making it one of the most commonly mutated GEF's in cancer (Fig. S6). We thus suspected that P-REX2a might be a

PTEN-regulating factor that is co-opted in tumors to stimulate PI3K signaling.

Thus the PREX2 nexus has been established and was known as early as 2009. The nexus with PTEN control is a major issue. The question may be if PREX2 mutations are stronger influences than say PTEN mutations.

There is also the issue regarding the melanoma cancer stem cell issue as well as we have been discussing elsewhere. Unlike a blood line stem cells or even prostate stem cells, the melanoma stem cell must most likely be a melanocyte, and one of the issues is how many melanocytes are stem in character, or is the stem cell not yet a melanocyte and if so what is it. A recent prior posting on prostate stem cells raises that issue as well.

I found one of the remarks especially compelling when the state:

In particular, we discovered that PREX2 mutations are both recurrent and functionally consequential in melanoma biology. Although its precise mechanism(s) of action remains to be elucidated in melanoma, PREX2 appears to acquire oncogenic activity through mutations that perturb or inactivate one or more of its cellular functions. This pattern of mutations may exemplify a category of cancer genes that is distinct from 'classic' oncogenes (often characterized by highly recurrent gain-of-function mutations) and tumour suppressors (inactivated by simple loss-of-function alterations). Instead, (over)expression of certain cancer genes with distributed mutation patterns may promote tumorigenicity either through dominant negative effects or more subtle dysregulation of normal protein functions

One other factor of interest was the calculation of mutation rates. They state:

This corresponded to an average mutation rate of 30 per Mb. However, the mutation rate varied by nearly two orders of magnitude across the 25 tumours . The acral melanomas showed mutation rates comparable to other solid tumour types (3 and 14 mutations per Mb), whereas melanomas from the trunk harboured substantially more mutations, in agreement with previous studies. In particular, sample ME009 exhibited a striking rate of 111 somatic mutations per Mb, consistent with a history of chronic sun exposure.

This is an interesting observation since it appears to confirm, albeit on this small sample, the impact of UV radiation, and I could argue radiation in general. Whether this gives additional merit to my prior work on X Ray scanners is still an open issue.

This is an interesting result and most likely will be followed by more detailed studies. There always are the issues regarding the clear causative nature and the details of the pathways.

5.5.12 Pleckstrin Homology

There has been some recent work (see De Semir et al) on the targeting of the Pleckstrin Homology, "PH", as an additional target for controlling melanomas. As DeSemir et al state regarding the Pleckstrin Homology Domain-Interacting Protein (PHIP) (slightly edited):

Given the important role of Akt in the IGF (Insulin Growth Factor) axis, we then assessed whether Phip was involved in Akt activation. …

Because of the uncharacterized role of PHIP in cancer, we performed cDNA microarray analysis to identify the global patterns of gene expression after suppression of Phip expression. Significance analysis of microarrays identified 51 down-regulated genes (including Igf2 and Tln1) and 184 overexpressed genes … Thus, PHIP can regulate the expression of upstream mediators of the IGF axis and downstream mediators of tumor cell invasion.

Having demonstrated Phip's functional role in promoting murine melanoma metastasis, we examined its impact on human melanoma progression.

We performed immunohistochemical analysis of PHIP expression on a tissue microarray cohort of 345 patients with primary cutaneous melanoma …

High levels of PHIP expression were found in each histological subtype of melanoma and accounted for almost one-third of the melanomas in this cohort.

High PHIP expression correlated significantly with the presence of ulceration, an adverse prognostic factor incorporated into the staging classification for melanoma whose biologic basis is poorly understood…

PHIP overexpression was significantly predictive of reduced distant metastasis-free survival … and disease-specific survival …

PHIP overexpression was an independent predictor of DMFS and DSS…

PHIP overexpression directly correlated with the progression of distant metastases, and with reduced survival, in both murine and human melanoma.

The human PHIP gene resides on the 6q14.1 locus. Deletions of the 6q arm have been shown in melanoma and have been suggested as a possible diagnostic marker. …

FISH analysis revealed that the PHIP locus was still present in all 78 melanomas examined.

Importantly, there was a significant correlation between PHIP copy number (assessed as a percentage of cells with three or more copies) and the corresponding PHIP immunohistochemical scores …

Melanomas with immunohistochemical scores of 1–3 had a significantly higher percentage of cells with increased copy number compared with melanomas with a PHIP score of 0 .. In addition, 80.6% of PHIP 3 melanomas had three or more copies of the PHIP locus.

Although we found no evidence of amplification, because PHIP copy number remains comparable with chromosome 6 centromeric copy number increased copy number of the PHIP melanomas for β-catenin mutations at six different sites (previously described in melanoma; COSMIC database) and found no mutations at any of these sites.

These results show that PHIP levels can be activated in a unique molecular subset of melanoma independent of mutations in these other four genes.

This brief summary of the work makes PHIP an interesting and attractive target. It presents a pathway element which is more a facilitator rather than a major participant (see Weinberg). As we shall note later from DeSemir et al, they contend that the PHIP target presents a more universal target especially for those melanomas which do not have well defined mutations in BRAF, NRAS or PTEN. As we have discussed previously, for example, PTEN mutations, loss of control in the Akt pathway, is often an end game in cancer progression, for example in prostate cancer and many others.

We will attempt to assemble some of the literature and present a brief summary of this area. In many ways it is distinct from the pathway targets themselves since the PH targets are smaller and often are found in many of the pathway elements. The PHD. Pleckstrin Homology Domain, has received significant interest by other researchers especially regarding its pathway control effects. For example Hirano et al have examined it in CML and Miyamoto et al in cardiology and the Akt pathway.

1.1.1.1 Pleckstrin and the Homology

We first examine Pleckstrin then its homology and its function. We begin first with Pleckstrin. Pleckstrin is a specific protein which is found in blood platelets. The name is derived using the concatenation of the phrases: **P**latelet and **LE**ukocyte **C K**inase substrate and the KSTR string of amino acids. It is located on 2p13.3.

Now the Pleckstrin Homology is defined as:

Pleckstrin homology domain (PH domain) is a protein domain which consists of approximately 120 amino acids. The PH domain is present in various proteins which are key elements of intracellular signaling as well as constituents of the cytoskeleton.

This domain can bind phosphatidylinositol lipids within biological membranes (such as phosphatidylinositol (3,4,5)-trisphosphate and phosphatidylinositol (4,5)-bisphosphate. PIP3 and PIP2), and proteins such as the βγ-subunits of heterotrimeric G proteins, and protein kinase C.

Through these interactions, PH domains play a role in recruiting proteins to different membranes, thus targeting them to appropriate cellular compartments or enabling them to interact with other components of the signal transduction pathways.

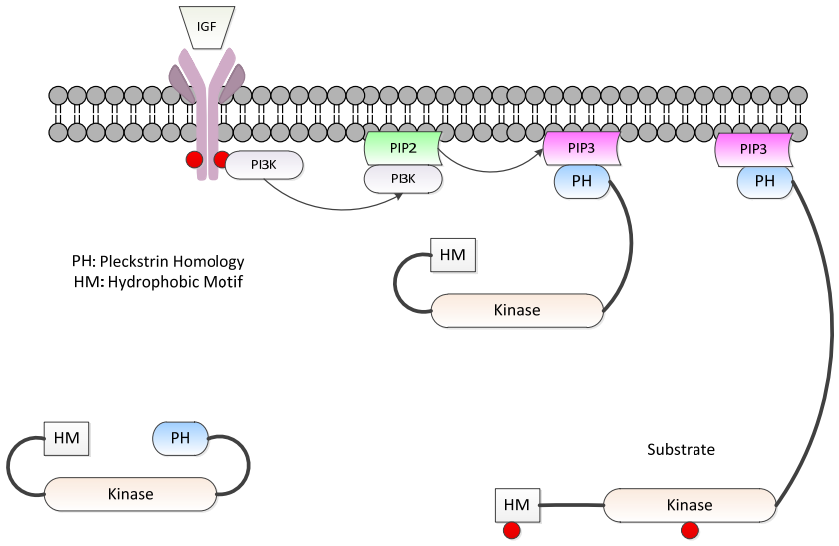
PH domains can be found in many different proteins, such as ARF. Recruitment to the Golgi in this case is dependent on both PtdIns and ARF. A large number of PH domains have poor affinity for phosphoinositides and are hypothesized to function as protein binding domains. Proteins reported to contain PH domains belong to the following families:

- Pleckstrin, the protein where this domain was first detected, is the major substrate of protein kinase C in platelets. Pleckstrin is one of the rare proteins to contain two PH domains.
- Ser/Thr protein kinases such as the Akt/Rac family, the beta-adrenergic receptor kinases, the mu isoform of PKC and the trypanosomal NrkA family.
- Tyrosine protein kinases belonging to the Btk/Itk/Tec subfamily.
- Insulin Receptor Substrate 1 (IRS-1).
- Regulators of small G-proteins like guanine nucleotide releasing factor GNRP (Ras-GRF) (which contains 2 PH domains), guanine nucleotide exchange proteins like vav, dbl, SoS and *S. cerevisiae* CDC24, GTPase activating proteins like rasGAP and BEM2/IPL2, and the human break point cluster protein bcr.
- Mammalian phosphatidylinositol-specific phospholipase C (PI-PLC) isoforms gamma

Discussion of PH in cancer is somewhat sparse and limited in detail. Bunz has a short reference (p 191) and Weinberg also has passing comments in several locations, and Schulz on p. 120.

1.1.1.2 PH and Pathways

The following is from Marks et al and shows how the PH domain can act as a binding and activating substrate in the overall pathway cascade process. It can unwrap from the complex protein of which it is a part, and then it can attach to a membrane protein and this allows activation, in the case below, by phosphorylating the resulting domain substrate. This simple model offers also a mechanism to block pathway activation as well.



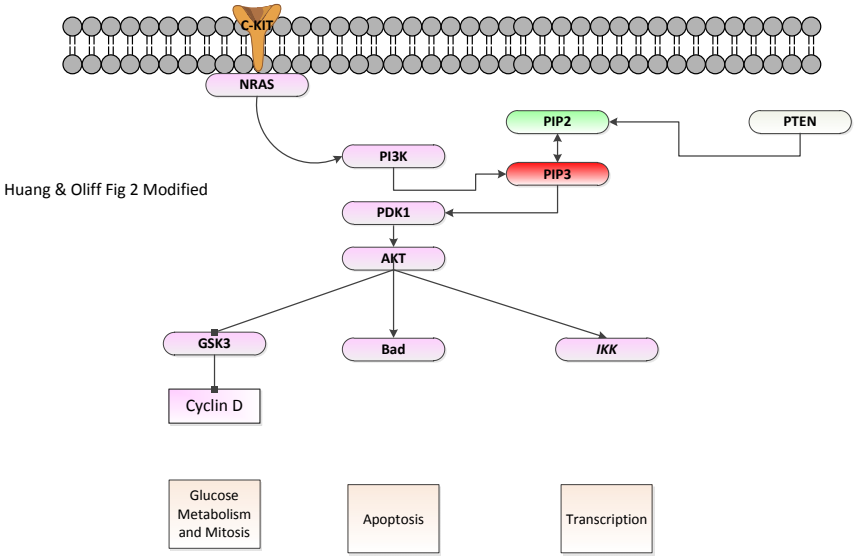
From: Marks et al, p 178.

As Huang and Oliff state regarding the PH domain:

There are three members of the AKT (PKB) family. They are widely expressed and implicated in apoptosis, insulin signalling and growth regulation. All three contain a pleckstrin lipid-binding domain (PH Domain)and are activated at the membrane by upstream kinases. Candidates for this upstream regulatory activity include integrin-linked kinase, PDK-1, and possibly AKT itself. In addition, AKT activity is regulated indirectly through modulation of lipid metabolism.

The loss of PTEN (a protein and lipid phosphatase) activity and the gain of PI3K (a protein and lipid kinase) activity correlate with AKT activity and binding of AKT to the membrane lipid, PI(3)P. The PI3K inhibitor wortmannin has already been shown to inhibit AKT signalling. Some proteins that have been shown to be substrates of AKT and relevant to apoptosis are listed. Antagonists of AKT kinase activity should inhibit signalling through these downstream effectors.

We demonstrate this pathway selectivity and control below. Here we have modified a Figure from Huang and Oliff to make the point that loss of PTEN control or over-activation of the Akt pathway can result in excess of proliferation and suppression of apoptosis. This is generalized below:



PTEN is a major control protein in pathway management. As Chow and Baker had stated in an earlier description of the effects of PTEN:

Soon after the discovery of its PIP3 phosphatase activity, PTEN was found to negatively regulate the PI3K/AKT pathway . Generation of PIP3 by growth factor-stimulated PI3K activity results in membrane recruitment of the serine–threonine kinase AKT via its pleckstrin homology (PH) domain, and activation by phosphoinositide-dependent kinases (PDK1 and 2) . Numerous AKT substrates have been identified affecting a broad range of cellular activities .

A few that have been implicated in oncogenic transformation include the Forkhead family of transcription factors (FOXO), $p27^{KIP1}$, MDM2, GSK3, BAD, IKK-b, and tuberin (TSC2), a negative regulator of mTOR. The specific targets phosphorylated by AKT vary with physiological stimuli and cell context and the mechanism for this selection is unclear. The complexity of this pathway is further underscored by the recent finding that mTOR can act both upstream and downstream of AKT activation. The raptor–mTOR complex can phosphorylate and activate AKT while the raptor–mTOR complex, which regulates growth and protein translation, can be activated downstream of AKT .

PTEN-mediated regulation of the PI3K/AKT pathway results in cell context-dependent effects on cell size, proliferation and survival. A dominant-negative form of AKT rescues the lethality caused by PTEN deficiency in flies. This strongly suggests that AKT is the major critical downstream target of PTEN activity ..

The impact of Akt has been understood now for quite a while and the BRAF facilitation when mutated has become a focal element of the control mechanism. However PH also plays a significant role and this too has been understood. As Dehaia states:

PI3-kinase triggers signaling through multiple pathways, many of which are thought to associate with cell growth and survival. PTEN, working in opposition to PI3-kinase, is therefore associated with cell death or arrest signals. Phospholipid residues such as PtdIns(3,4,and 5)P_3

are present in cells upon stimulation by several growth factors, such as platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), and epidermal growth factor (EGF).

Upon activation by growth factor, proteins containing a pleckstrin-homology (PH) domain are recruited to the membrane 3 where they associate with phospholipids. One of the PH domain-containing proteins relevant in this pathway is the serine-threonine kinase, AKT, also known as PKB or RAC1*. AKT, in turn, and as a consequence of lipid binding, alters its conformation to allow two of its residues, threonine 308 and serine 473, to be phosphorylated and therefore become active.*

The kinase responsible for phosphorylation of threonine 308 is phosphonositide-dependent kinase 1 (PDK1), an enzyme which also contains a PH domain and is therefore dependent on lipid binding for its full activity. There is some preliminary evidence, predominantly from in vitro studies, that a second lipid-dependent, PH domain-containing enzyme, ILK (integrin-linked kinase), is responsible for phosphorylation of the serine 473.

Further, a recent paper has proposed that the kinase responsible for Ser 473 phosphorylation might in fact be PDK1, when it associates with certain specific proteins, such as PDK1 interacting fragment (PIF), as seen by in vitro studies. By dephosphorylating D3 residues on PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2, PTEN works in opposition to the PI3K/AKT pathway and therefore counteracts cell survival mechanisms elicited by this signaling. The mechanisms of cell survival associated with AKT appear to involve multiple pathways, including growth factors, cytokines, c-myc overexpression, UV irradiation, and matrix detachment.

One of the known signals activated by AKT is its phosphorylation of the Bcl-2 family member, BAD: phosphorylation of BAD results in suppression of apoptosis. AKT has also been reported to counteract the apoptotic response of several cellular factors. Recently, the transcription factor NF-kappaB has been implicated in the apoptotic response antagonized by the PI3K/AKT pathway

Thus we have demonstrated that PH activateable proteins such as Akt can be deactivated if it were possible to focus on the PH Domain as a target sector. Recent work has demonstrated that in some detail.

1.1.1.3 Current Understanding

We now will examine some of the current understanding of PH and its implications in melanoma specifically. We examine the work of two other groups and then readdress that of DeSemir et al.

As Farang Fallah et al state:

As a major substrate of the insulin receptor, insulin receptor substrate 1 (IRS-1) plays a central role in transducing insulin-dependent signals that regulate biological processes such as cell growth and cellular uptake of glucose. IRS-1 is a modular protein comprised of an N-terminal region harboring a pleckstrin homology (PH) domain, followed by a phosphotyrosine binding (PTB) domain that cooperatively ensures selective recognition and efficient substrate

phosphorylation by the activated insulin receptor (IR). The C-terminal portion contains multiple tyrosine phosphorylation motifs which serve as docking sites for the recruitment of various SH2 (Src-homology 2) domain containing signaling molecules, such as phosphatidylinositol 3-kinase (PI 3-kinase), Grb-2 adaptor protein, and SHP2 (SH2 containing phosphatase 2) tyrosine phosphatase, which in turn elicit the activation of biochemical cascades that promote the metabolic and growth responses to insulin….

In the present study we demonstrate that overexpression of either PHIP or IRS-1 alone in muscle cells was not sufficient in promoting transport of GLUT4 to plasma membrane surfaces This is consistent with other observations, indicating that activation of IRS-1-associated signaling effectors such as PI 3-kinase, although necessary, is not sufficient for GLUT4 activation.

Notably, growth factors such as platelet-derived growth factor and interleukin-4 can activate PI 3-kinase as efficiently as insulin and yet fail to stimulate glucose transport in insulinsensitive cells (17, 22).

One possible explanation is that additional PHIP/IRS-1/PI 3-kinase-independent pathways are required to coordinate GLUT4 intracellular routing. Indeed, recent evidence points to a novel insulin-responsive pathway that recruits flotillin/CAP/CBL complexes to IR-associated lipid rafts in the plasma membrane, an event which is thought to potentiate GLUT4 docking to the cell surface after IR activation.

Our data, however, provide support for the involvement of PHIP/IRS-1 complexes in glucose transporter GLUT4 translocation in muscle cells. Specifically, the use of DN-PHIP or IRS-1 PH domain constructs known to interfere with efficient IR–IRS-1 protein interaction, and hence productive signal transduction from IRS-1 to PI 3-kinase, blocked the ability of insulin to stimulate GLUT4 mobilization in L6 myoblasts and inhibited insulin-stimulated actin cytoskeletal reorganization, a process required for the productive incorporation of GLUT4 vesicles at the cell surface. Moreover, this inhibition did not coincide with changes in the autophosphorylation status of the IR.

As Barnett et al state:

Akt/PKB (protein kinase B) is a serine/threonine kinase which has a key role in the regulation of survival and proliferation [1–8]. There are three isoforms of human Akt (Akt1, Akt2 and Akt3) and they all have an N-terminal PH (pleckstrin homology) domain and a kinase domain separated by a 39-amino-acid hinge region. The PH domains have approx. 60% identity and the kinase domains are >85% identical.

The hinge region is the least conserved at approx. 28% identity. The Akt active-site residues, described in a recent report on the crystal structure of Akt2 containing an ATP analogue and a peptide substrate , are the same in all three iso-enzymes. Based on the high degree of homology between the AGC protein kinase family members, the identification of specific active-site inhibitors has been predicted to be difficult. The identification of Akt iso-enzyme-specific inhibitors seemed to be an even greater challenge….

Two Akt inhibitors were identified that exhibited isoenzyme specificity. The first compound (Akt-I-1) inhibited only Akt1 while the second compound (Akt-I-1,2) inhibited both Akt1 and Akt2 with IC_{50} values of 2.7 and 21 μM respectively. Neither compound inhibited Akt3 nor mutants lacking the PH (pleckstrin homology) domain at concentrations up to 250 μM.

These compounds were reversible inhibitors, and exhibited a linear mixed-type inhibition against ATP and peptide substrate. In addition to inhibiting kinase activity of individual Akt isoforms, both inhibitors blocked the phosphorylation and activation of the corresponding Akt isoforms by PDK1 (phosphoinositide-dependent kinase 1).

A model is proposed in which these inhibitors bind to a site formed only in the presence of the PH domain. Binding of the inhibitor is postulated to promote the formation of an inactive conformation. In support of this model, antibodies to the Akt PH domain or hinge region blocked the inhibition of Akt by Akt-I-1 and Akt-I-1,2. These inhibitors were found to be cell-active and to block phosphorylation of Akt at Thr_{308} and Ser_{473}, reduce the levels of active Akt in cells, block the phosphorylation of known Akt substrates and promote TRAIL (tumour-necrosis-factor-related apoptosis inducing ligand)-induced apoptosis in LNCap prostate cancer cells.

We can now return to the results of DeSemir et al. As they look to the usefulness of PHIP they state:

Although melanomas with mutant v-Raf murine sarcoma viral oncogene homolog B1 (BRAF) can now are effectively targeted, there is no molecular target for most melanomas expressing wildtype BRAF. Here, we show that the activation of Pleckstrin homology domain-interacting protein (PHIP), promotes melanoma metastasis, can be used to classify a subset of primary melanomas, and is a prognostic biomarker for melanoma.

Systemic, plasmid based shRNA targeting of Phip inhibited the metastatic progression of melanoma, whereas stable suppression of Phip in melanoma cell lines suppressed metastatic potential and prolonged the survival of tumor-bearing mice.

The human PHIP gene resides on 6q14.1, and although 6q loss has been observed in melanoma, the PHIP locus was preserved in melanoma cell lines and patient samples, and its overexpression was an independent adverse predictor of survival in melanoma patients. In addition, a high proportion of PHIP-overexpressing melanomas harbored increased PHIP copy number.

PHIP-overexpressing melanomas include tumors with wild-type BRAF, neuroblastoma RAS viral (v-ras) oncogene homolog, and phosphatase and tensin homolog, demonstrating PHIP activation in triple-negative melanoma. These results describe previously unreported roles for PHIP in predicting and promoting melanoma metastasis, and in the molecular classification of melanoma.

This demonstrates the extended ability of PHIP to enhance the usefulness of other markers. They continue as follows:

As a result, "triple-negative melanoma" patients, whose tumors harbor wild-type v-Raf murine sarcoma viral oncogene homolog B1 (BRAF), neuroblastoma RAS viral (vras) oncogene homolog (NRAS), and phosphatase and tensin homolog (PTEN) (the most common mutations observed in melanoma), are not candidates for most targeted therapies developed to date.

This as we have noted before is one of the most significant findings. We know that BRAF mutations are currently targeted with some beneficial albeit temporally limited results. Perhaps PHIP may add an additional targeting.

They conclude:

Overexpression or mutation of genes that play important roles in tumor progression. A high proportion of melanomas are characterized by BRAF, NRAS, or PTEN mutations. However, the molecular basis of triple-negative melanomas lacking these mutations is poorly characterized. Our results suggest that PHIP levels may be used to classify some melanomas that lack these three mutations. It is likely that additional molecular aberrations will be identified to further characterize triple-negative melanomas.

Along with recent studies demonstrating that the IGF axis is activated in melanomas with acquired resistance to BRAF inhibition (23), these studies have identified IGF signaling as an important alternative pathway to promote melanoma progression. Overall, our studies identify PHIP as a molecular mediator of melanoma progression that also appears to function in the setting of a subset of triple-negative melanomas.

Clearly BRAF, NRAS and PTEN mutations are well defined targets, BRAF especially for melanoma and PTEN seems to span a wide number of cancers. However if they are not changed the PHIP mutation seems more in line with wit an reasonable target.

5.5.13 Crosstalk

As is well known now the BRAF mutation found in certain melanomas can be somewhat controlled via the use of vemurafenib. However and possibly surprisingly there is an increase in other cancers.

Su et al conclude:

Mutations in RAS, particularly HRAS, are frequent in cutaneous squamous-cell carcinomas and keratoacanthomas that develop in patients treated with vemurafenib. The molecular mechanism is consistent with the paradoxical activation of MAPK signaling and leads to accelerated growth of these lesions.

Pathways have cross talk, and when one pulls one string another may also be pulled. The authors further note:

The t→a transversion at position 1799 of BRAF (BRAF V600E) is present in approximately 50% of patients with metastatic melanoma.1,2 BRAF V600E induces constitutive signaling through

the mitogen-activated protein kinase (MAPK) pathway, stimulating cancer-cell proliferation and survival.2 The clinical development of inhibitors of oncogenic BRAF, termed type I BRAF inhibitors, which block the active conformation of the BRAF kinase, has led to a high rate of objective tumor responses and improvement in overall survival, as compared with standard chemotherapy.3-5 However, nonmelanoma skin cancers — well-differentiated cutaneous squamous-cell carcinomas and keratoacanthomas — have developed in approximately 15 to 30% of patients treated with type I BRAF inhibitors such as vemurafenib and dabrafenib.

This may open a door to several new approaches. First understanding pathways better and deducing the effects on blocking one of the paths, and multi-drug analysis.

Su et al conclude:

In the skin carcinogenesis model, the BRAF inhibitor PLX4720 drove paradoxical activation of the MAPK pathway and proliferation of HRAS Q61Ltransformed keratinocytes, with decreased latency and accelerated growth of cutaneous squamous cell carcinomas and keratoacanthomas. PLX4720 was not itself a true tumor promoter because it could not substitute for TPA. Instead, PLX4720 accelerated the growth of preexisting RAS-mutant lesions.

Taken together with the clinical observations and functional analyses, our data provide circumstantial evidence to suggest that vemurafenib does not initiate tumorigenesis but rather accelerates the progression of preexisting subclinical cancerous lesions with strong upstream MAPK signaling potential.

These findings explain why the lesions generally develop early after vemurafenib treatment and only in a subset of patients. In conclusion, our data provide a molecular mechanism for the development of clinical toxicity that is the opposite of what would be expected from a targeted oncogene inhibitor. This mechanism accounts for the development of cutaneous squamous-cell carcinomas and keratoacanthomas, notably of the skin, but it is not clear whether it is relevant to the development of squamous-cell carcinomas in other organs.
]
Our findings support the caution against investigating single-agent type I BRAF inhibitors in patients with cancers driven by RAS or by activated receptor tyrosine kinases.

The discovery that the development of these lesions is driven by RAS and by MAPK in patients receiving BRAF inhibitors, as well as the effects noted in the animal model, point to the usefulness of combining a BRAF inhibitor with a MEK inhibitor to prevent this toxic effect[34] and make way for the clinical development of a new generation of BRAF inhibitors selected to avoid paradoxical MAPK-pathway activation.

The main conclusion is that treating a single pathway element may not cure the problem. Namely there is considerable crosstalk and that this crosstalk must be understood as one develops protocols for effective treatment.

5.6 TRANSCRIPTION ELEMENTS

We now want to consider transcription elements. These are critical factors since it is the transcription of DNA to RNA that leads to the over expression or even under expression of certain genes that result in loss of homeostasis.

The following is a list of significant transcription factors that we consider in melanoma. We shall focus on a subset of these.

- 4EBP1
- CCND1
- CDK4
- CREB
- eIF4E
- ETV1
- FOS
- GLI2
- HIF
- INK4A
- JUN
- LEF
- MITF
- MYC
- NF-κB
- TCF
- TCF
- TOR

5.6.1 cMYC

Myc or specifically c-Myc, is a powerful gene element which induces cell growth. c-Myc is so strong promoter of cell proliferation and growth. c-Myc is a transcription factor which is essential in the growth and expansion of the cell.

In the paper by Iwata et al the authors examine its influence during the development of PIN. They state:

Lo-MYC and Hi-MYC mice develop prostatic intraepithelial neoplasia (PIN) and prostatic adenocarcinoma as a result of MYC overexpression in the mouse prostate[1]. However, prior studies have not determined precisely when, and in which cell types, MYC is induced. Using immunohistochemistry (IHC) to localize MYC expression in Lo-MYC transgenic mice, we show that morphological and molecular alterations characteristic of high grade PIN arise in luminal epithelial cells as soon as MYC overexpression is detected.

These changes include increased nuclear and nucleolar size and large scale chromatin remodeling. Mouse PIN cells retained a columnar architecture and abundant cytoplasm and

appeared as either a single layer of neoplastic cells or as pseudo-stratified/multilayered structures with open glandular lumina—features highly analogous to human high grade PIN.

Also using IHC, we show that the onset of MYC overexpression and PIN development coincided precisely with decreased expression of the homeodomain transcription factor and tumor suppressor, Nkx3.1. Virtually all normal appearing prostate luminal cells expressed high levels of Nkx3.1, but all cells expressing MYC in PIN lesions showed marked reductions in Nkx3.1, implicating MYC as a key factor that represses Nkx3.1 in PIN lesions.

To determine the effects of less pronounced overexpression of MYC we generated a new line of mice expressing MYC in the prostate under the transcriptional control of the mouse Nkx3.1 *control region. These ''Super-Lo-MYC'' mice also developed PIN, albeit a less aggressive form. We also identified a histologically defined intermediate step in the progression of mouse PIN into invasive adenocarcinoma. These lesions are characterized by a loss of cell polarity, multi-layering, and cribriform formation, and by a ''paradoxical'' increase in Nkx3.1 protein. Similar histopathological changes occurred in Hi-MYC mice, albeit with accelerated kinetics.*

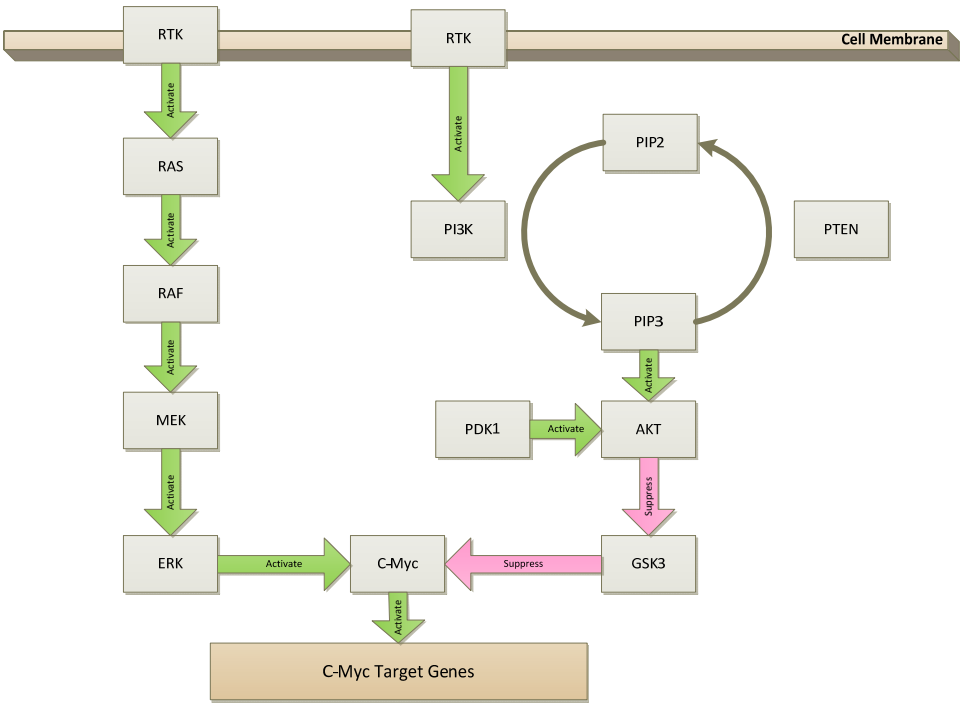
Our results using IHC provide novel insights that support the contention that MYC overexpression is sufficient to transform prostate luminal epithelial cells into PIN cells in vivo. *We also identified a novel histopathologically identifiable intermediate step prior to invasion that should facilitate studies of molecular pathway alterations occurring during early progression of prostatic adenocarcinomas.*

In the following graphic we depict the influence elements on c-Myc. This is a complex system of interlinking genes which when expressed in the correct manner can slow cell over expansion. The chart below is a modification from Bunz (p. 203) and it shows the gross characteristics of this control path. PTEN is a key element in control. What this does not show are two key elements, and indirectly a third.

First it does not show the fact that these are protein concentrations at work, one influencing the other and so forth. There is a feedback mechanism missing.

Second, it does not portray the temporal elements, namely this is a static gross representation of the influencing factors as if done in some generic snapshot. I fact the concentrations are time varying and it is this time variation which when combined with the feedback loops renders certain instabilities leading to malignancy, namely uncontrolled growth.

Third, it fails to show the other genes and specifically the feedback mechanism of these genes. Namely PTEN is influence by these.



As Deutsch et al state:

MYC is one of the earliest genes to be expressed in the prostate gland after androgen stimulation. Transgenic mice that overexpress Myc develop prostate neoplasia. The importance of MYC overexpression in prostate cancer has been corroborated by the discovery of an increased copy number of the portion of 8q containing MYC in prostate-cancer samples, and particularly in advanced cases.

Only 6% of primary tumours show a gain of 8q sequences, whereas this change is seen in 89% of tumor recurrences after hormonal therapy.
MYC (a downstream target of AR) has been shown to be regulated by the AR and to be needed for AR-dependent and AR-independent growth. These data suggest that MYC might be involved in the development of androgen-independent prostate cancer, including that resulting from an increase in AR signalling.

5.6.2 MITF

MITF (microthalmia associated transcription factor) is a mediator of the pigmentation response in melanocytes (see Hearing and Leong p 55). It is also thought that the transcription of the MITF gene is facilitated by multiple transcription factors. MITF is both a transcription factor itself as well as a pathway mediator as we shall demonstrate. It functions in both the nucleus and the cytoplasm. MITF is required for the development, maintenance and survival of the melanocyte. It has been argued that MITF is one of the gene products that allow melanoma to survive the attack by normal chemotherapy. As in Hearing and Leong, p 61, the dysregulation of transcription factors is putatively the prime reason for cancer. MITF dysregulation is one of these transcription factors.

As Yokoyama et al state:

So far, two genes associated with familial melanoma have been identified, accounting for a minority of genetic risk in families. Mutations in CDKN2A account for approximately 40% of familial cases[1], and predisposing mutations in CDK4 have been reported in a very small number of melanoma kindreds. Here we report the whole-genome sequencing of probands from several melanoma families, which we performed in order to identify other genes associated with familial melanoma. We identify one individual carrying a novel germline variant … in the melanoma-lineage-specific oncogene microphthalmia-associated transcription factor (MITF). …

Consistent with this, the E318K variant was significantly associated with melanoma in a large Australian case–control sample. Likewise, it was similarly associated in an independent case–control sample from the United Kingdom. In the Australian sample, the variant allele was significantly overrepresented in cases with a family history of melanoma, multiple primary melanomas, or both. The variant allele was also associated with increased nevus count and non-blue eye colour. Functional analysis of E318K showed that MITF encoded by the variant allele had impaired simulation and differentially regulated several MITF targets. These data indicate that MITF is a melanoma predisposition gene and highlight the utility of whole-genome sequencing to identify novel rare variants associated with disease susceptibility.

This identification of a mutated MITF and familial melanoma is a clear indication of the power that MITF has in establishing melanoma in general.

As Wellbrock and Marais state:

Melanocytes are pigmented skin cells that protect us from ultraviolet radiation. The processes regulating melanocyte differentiation are intensely studied because melanocytes are thought to be the precursors of melanoma, a skin cancer whose incidence is increasing in Western societies. A master regulator of melanocyte differentiation is the microphthalmia-associated transcription factor (MITF). Strikingly, MITF levels are reduced in spontaneously transformed melanocytes, and low MITF expression correlates with poor prognosis in melanoma.

MITF regulation is complex. For example, the differentiation factor melanocyte stimulating hormone strongly increases its expression in a cAMP and cAMP response element binding protein (CREB) transcription factor–dependent manner. Another signaling module that regulates MITF is the RAS–RAF–MEK–ERK signaling cascade, which acts downstream of the receptor tyrosine kinase cKIT to stimulate MITF phosphorylation on serine 73 (S73) and enhances its transcriptional activity.

However, extracellular regulated protein kinase (ERK)–mediated S73 phosphorylation also targets MITF for ubiquitin-dependent degradation through the proteasome pathway. There are three RAS (H-RAS , K-RAS , and N-RAS) and three RAF (A-RAF , B-RAF , and C-RAF) genes in humans. N-RAS is mutated in 5–20% of melanomas, and B-RAF is mutated in 50–70% of melanomas. The most common mutation in B-RAF (90%) is a glutamic acid for valine

substitution at position 600, which produces a highly active kinase that stimulates constitutive ERK signaling and stimulates melanoma cell proliferation and survival.

In this study, we show that V600E B-RAF triggers MITF degradation in mouse and human melanocytes and that its re-expression inhibits proliferation. Furthermore, MITF up-regulation suppresses melanoma cell proliferation.

These data suggest that high MITF levels are anti-proliferative, and, therefore, its expression must be suppressed for transformation by oncogenic B-RAF.

The identification of V600E B-RAF triggering of MITF degradation is a powerful observation. The actual mechanism may not be fully understood but the causal basis is compelling. It is this type of cascade behavior that must be considered in such changes. The final conclusion is also compelling. MITF must be suppressed either by mutation or as seen here by suppression by another mutated gene. They conclude:

MITF re-expression in B-RAF–transformed melanocytes inhibits their proliferation. Furthermore, differentiation-inducing factors that elevate MITF expression in melanoma cells inhibit their proliferation, but when MITF up-regulation is prevented by RNA interference, proliferation is not inhibited. These data suggest that MITF is an anti-proliferation factor that is down-regulated by B-RAF signaling and that this is a crucial event for the progression of melanomas that harbor oncogenic B-RAF.

As Miller and Mihm state:

Mice lacking functional MITF are albino because they lack melanocytes, whereas those with partial MITF function have premature graying owing to the death of melanocytes. These experiments show that MITF is important in the differentiation and maintenance of melanocytes.

MITF appears to contribute to melanocyte survival by increasing the expression of the BCL-2 *gene, a key antiapoptotic factor.*[59] *In mice, deficiencies of both MITF and BCL-2 cause gray hair due to a loss of differentiated melanocytes. The loss of melanocytes is due to the apoptosis of melanocyte progenitor cells in the hair follicle.*

In melanoma cell lines, a reduction in BCL-2 protein also causes cell death, suggesting that the survival of malignant melanocytes depends on BCL-2. … MITF functions in a key pathway leading to melanocyte pigmentation. Intracellular signaling induced by α-MSH acting on MC1R increases MITF expression, which in turn increases the transcription of genes underlying melanin synthesis: tyrosinase, tyrosinase-related-protein 1, and dopachrome tautomerase.

*MITF also regulates the transcription of the melanocyte-specific genes silver homologue (*SILV*) and melan-A (*MLANA*), whose immunohistochemical detection points to the diagnosis of melanoma. In addition, MITF causes cell-cycle arrest by the induction of INK4A.*

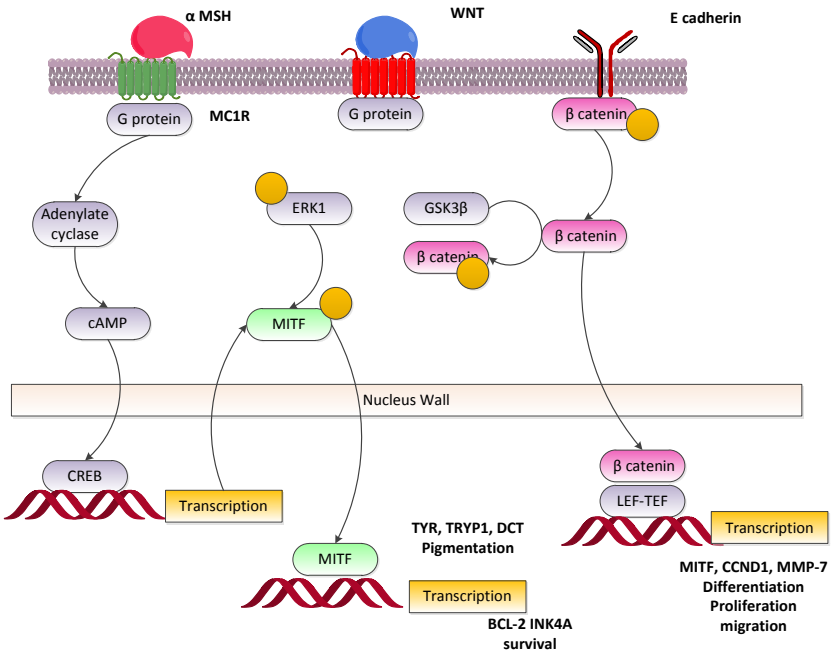
Decreased or absent pigmentation and decreased or absent expression of SILV and MLANA accompany the progression from nevus to melanoma.

*Tumors that are deficient in these proteins have a poor prognosis. Expression of the melastatin 1 (*TRPM1*) gene, whose function is unknown, is also controlled by MITF. Melanomas that are deficient in melastatin have a poor prognosis. The mechanism of decreased expression of these genes is a puzzle because MITF is present in nearly all melanomas.[71-73] Although MITF causes differentiation and cellcycle arrest in normal melanocytes, melanoma cells do not have these characteristics.*

Recently, a large-scale search for genomic changes in melanoma with the use of high-density single-nucleotide polymorphisms (SNPs) found an increased copy number (4 to 119 copies per cell) of a region of chromosome 3 that includes the MITF *locus. This increase was accompanied by the increased expression of MITF protein. The overexpression of both MITF and BRAF could transform primary cultures of human melanocytes, implicating MITF as an oncogene.*

Notably, MITF amplification occurs most frequently in tumors that have a poor prognosis and is associated with resistance to chemotherapy.[74] Interference with MITF function increased the chemosensitivity of a melanoma cell line, making MITF a potential target for treatment.

Miller and Mihm depict the MITF functions in the following Figure (as modified):



They state:

In the MITF pathway, MITF is regulated at both transcriptional and post-translational levels.

The post-translational activation can occur through the ERK component of the MAPK pathway.

The chief transcriptional pathways that are activated by extracellular signals are the melanocortin and WNT pathways.

The melanocortin pathway regulates pigmentation through the MC1R. MC1R activates the cyclic AMP (cAMP) response-element binding protein (CREB).

Increased expression of MITF and its activation by phosphorylation (P) stimulate the transcription of tyrosinase (TYR), tyrosinase-related protein 1 (TYRP1), and dopachrome tautomerase (DCT), which produce melanin; melan-A, silver homologue, and melastatin 1 (TRPM1) are melanoma markers; inhibitor of kinase 4A (INK4A) leads to cell-cycle arrest, and BCL-2 suppresses apoptosis.

In the β-catenin pathway, β-catenin plays a central role in cell adhesion and cell signaling. Signals from WNT ligands block the breakdown of β-catenin. When WNT proteins bind the G-protein–coupled receptor (called frizzled), they inactivate the kinase GSK3β, an enzyme that phosphorylates β-catenin and targets it for destruction in the proteosome.

Then β-catenin accumulates in the cytoplasm and translocates to the nucleus, where it binds to LEF–TCF transcription factors and increases the expression of several genes, including MITF, the cell-cycle mediator cyclin D1 (CCND1), and matrix metalloproteinase 7 (MMP-7).

Further, regarding the relationship with other pathway elements, Liu et al state:

As a survival factor for melanocytes lineage cells, MiTF plays multiple roles in development and melanomagenesis. What role MiTF plays in the DNA damage response is currently unknown. In this report we observed that MiTF was phosphorylated at serine 73 after UVC radiation, which was followed by proteasome-mediated degradation.

Unlike after c-Kit stimulation, inhibiting p90RSK-1 did not abolish the band shift of MiTF protein, nor did it abolish the UVC-mediated MiTF degradation, suggesting that phosphorylation on serine 73 by Erk1/2 is a key event after UVC. Furthermore, the MiTF-S73A mutant ...was unable to degrade and was continuously expressed after UVC exposure.

Compared to A375 melanoma cells expressing wildtype MiTF (MiTF-WT), cells expressing MiTF-S73A mutant showed less p21 $^{WAF1/CIP1}$ *accumulation and a delayed p21*$_{WAF1/CIP1}$ *recovery after UVC. Consequently, cells expressing MiTF-WT showed a temporary G1 arrest after UVC, but cells expressing MiTF-S73A mutant or lack of MiTF expression did not. Finally, cell lines with high levels of MiTF expression showed higher resistance to UVC-induced cell death than those with low-level MiTF.*

These data suggest that MiTF mediates a survival signal linking Erk1/2 activation and p2 $^{WAF1/CIP1}$ *regulation via phosphorylation on serine 73, which facilitates cell cycle arrest. In addition, our data also showed that exposure to different wavelengths of UV light elicited different signal pathways involving MiTF.*

This demonstrates that UVC does have substantial mitogenic effects and may be a possible model for the mutation process.

Bourneuf et al state:

The incidence of cutaneous melanoma, the most aggressive form of skin cancer, is growing every year worldwide. Although most of the cases are sporadic and likely due to UV exposure, around 10% occur on a familial setting, and many studies have been performed to identify genetic variants conferring susceptibility to this type of cancer.

The familial setting is a powerful means to identify gene mutations that are germ line. The authors continue:

Two high-risk genes have been discovered in melanoma prone kindred, namely, CDKN2A and CDK4, both involved in cell cycle regulation through the p53/Rb pathway.

We will focus on both of these. Remember that CDK4 is a cyclin dependent kinase and plays a critical role in the cell cycle and mitotic change.

So far, the other genes, the variants of which are associated with melanoma, have been considered low-risk genes and are involved mostly in pigmentation, an important risk factor with a higher incidence of melanoma in fair-skinned patients.

For example, the melanocortin 1 receptor (MC1R) gene has been shown to enhance the penetrance of the CDKN2A mutations in patients. Its effect on melanoma, although it is also suspected to be related to UV sensitivity via unknown mechanisms, is due mainly to its major involvement in skin and hair pigmentation. Recent genome-wide association studies focusing on melanoma and number of nevi highlighted the potential role of several other genes such as MTAP (methylthioadenosine phosphorylase) and TYR (tyrosinase), which are also involved in pigmentation.

They then continue:

Numerous other genes have been shown to affect melanoma biology, but their involvement as predisposing loci for melanoma remains unelucidated.

One of these genes, MITF, is considered a master regulator of melanocyte function, including development, migration, survival, and differentiation, through complex mechanisms of regulation. Recently, MITF has been shown to be responsible for the melanocyte lineage specificity of DICER transcriptional regulation, thus contributing to melanocyte differentiation. In melanoma, Garraway et al. identified MITF as a lineage-specific oncogene, of which amplification in 10–20% of the melanoma samples was correlated with decreased patient survival.

Also, Giuliano et al. demonstrated that MITF was preventing melanoma cells' senescence through a DDR/p53 signaling pathway.

In addition, somatic mutations of MITF were described in a fraction of primary tumors and metastasis. This gene therefore plays a major dual role between differentiation of melanocytes and proliferation of melanoma cells.

Thus the presence of a mutation in MITF as we have discussed is a significant factor. Clearly MITF as a transcription factor has a significant role in over production and as a pathway element can enhance such over-expression.

5.6.3 NF-κB

NF-κB is another transcription protein seen in melanoma. This protein is characterized by:

- NF-κB is a transcription factor that resides in the cytoplasm.
- It is called Nuclear Factor and was identified by David Baltimore as an enhancer factor for the κ chain of Ig light chain in B lymphocytes
- When activated it moves to the nucleus and is a transcription factor in activating over 400 genes
- It is activated by a large number of stimuli and its action of a large gene set causes significant DNA activity
- NF-κB appears on 10q24 and is somatic and acts in a dominant manner.

NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) is a protein complex that controls the transcription of DNA. NF-κB is widely used by eukaryotic cells as a regulator of genes that control cell proliferation and cell survival. As such, many different types of human tumors have mis-regulated NF-κB: that is, NF-κB is constitutively active. Active NF-κB turns on the expression of genes that keep the cell proliferating and protect the cell from conditions that would otherwise cause it to die via apoptosis.

As Amiri and Richmond state:

Nuclear Factor-kappa B (NF-κB) is an inducible transcription factor that regulates the expression of many genes involved in the immune response. Recently, NF-κB activity has been shown to be upregulated in many cancers, including melanoma. Data indicate that the enhanced activation of NF- κB may be due to deregulations in upstream signaling pathways such as Ras/Raf, PI3K/Akt, and NIK. Multiple studies have shown that NF-κB is involved in the regulation of apoptosis, angiogenesis, and tumor cell invasion, all of which indicate the important role of NF-κB in tumorigenesis. Thus, understanding the molecular mechanism of melanoma progression will aid in designing new therapeutic approaches for melanoma.

They continue:

Constitutive activation of NF-κB is an emerging hallmark of various types of tumors including breast, colon, pancreatic, ovarian, and melanoma [9–14]. In the healthy human, NF-κB regulates the expression of genes involved in normal immunologic reactions (e.g. generation of immunoregulatory molecules such as antibody light chains) in response to proinflammatory

cytokines and by-products of microbial and viral infections [15–17]. NF-κB also modulates the expression of factors responsible for growth as well as apoptosis. However, increased activation of NF-κB results in enhanced expression of proinflammatory mediators, leading to acute inflammatory injury to lungs and other organs, and development of multiple organ dysfunctions as well as cancer.

They then summarize NF-kB's role in melanoma as:

3.1. Apoptosis resistance and cell proliferation*: In processes such as tumor initiation and promotion where prolonged survival of cells is a crucial event, NF-κB plays an important role as a mediator of inhibition of apoptosis. In melanoma, NF-κB has been shown to activate expression of anti-apoptotic proteins such as tumor necrosis factor receptor-associated factor 1 (TRAF1), TRAF2, and the inhibitor-ofapoptosis (IAP) proteins c-IAP1, c-IAP2, and melanoma inhibitor of apoptosis (ML-IAP), survivin as well as Bcl-2 like proteins…*

3.2. Invasion and metastasis*: In invasion and metastasis of melanoma, NF-κB may regulate the production of prostaglandins via cyclooxygenase-2 (COX-2), which has been shown to be overexpressed in melanoma [44,45]. It was shown that COX-2 is expressed in the majority of primary malignant melanoma, as well as in five human malignant melanoma cell lines….*

However as Liu et al (2006) state:

Malignant melanoma is the most lethal skin cancer, whose ability to rapidly metastasize often prevents surgical cure.

Furthermore, the systemic treatment of melanoma is largely ineffective due to the intrinsic resistance of melanoma cells to numerous anticancer agents. Increased survival of melanoma cells is primarily attributed to the constitutive activation of the transcription factor nuclear factor kB (NF-kB), which regulates the expression of many anti-apoptotic, pro-proliferative and pro-metastatic genes.

Canonical activation of the NF-kB pathway occurs when NF-kB switches its localization from the cytoplasm, where it is maintained inactive by assembly with the inhibitor IkB protein, to the nucleus, where NF-kB regulates gene expression. NF-kB activation relies upon the phosphorylation dependent ubiquitination and degradation of IkB mediated by the IkB kinase (IKK) complex and b-Trcp E3 ubiquitin ligases.

Consequently, both IKK activity and the levels of b-Trcp regulate the extent of IkB degradation and hence NF-kB activation. The genetic basis that underlies the elevated NF-kB activity in malignant melanoma largely remains elusive.

Constitutively active IKK has been demonstrated to sustain NF-kB activation in human melanoma cells, resulting in induction of the chemokine CXCL1. CXCL1, in turn, is capable of activating IKK and NF-kB and promoting cell survival and tumorigenesis …. However, the original genetic alterations that initiate this feed-forward mechanism in melanoma remain unclear.

One of the major oncogenic events described in the genesis of malignant melanoma is constitutive activation of the Ras-regulated RAF-MEK-ERK mitogen-activated protein kinase (MAPK) pathway. This is achieved most frequently by activating mutations in either BRAF (e.g. V600E substitution) or, less frequently, in N-RAS … Recent evidence indicates that oncogenic BRAF activity is essential for human melanoma cell growth and survival …

However, despite prior reports that RAF can activate NF-kB …, the mechanism(s) by which $BRAF_{V600E}$ ($BRAF_{VE}$) may elicit NF-kB signaling in melanoma cells have not yet been elucidated. Activation of the canonical NF-kB pathway depends on both IKK activity, which has been shown to be elevated in human melanomas….

Liu et al conclusion is speculative but telling:

Taken together, these data support a model in which mutational activation of BRAF in human melanomas contributes to constitutive induction of NF-κB activity and to increased survival of melanoma cells.

Again we have the issue of speculation as to where and why the mutations occur. Here they speculate about the BRAF mutation resulting in the antiapoptotic control with NF-κB.

5.7 MICRO RNAS AND EPIGENETIC FACTORS

Epigenetic modifications are considered hereditable changes in gene expression occurring when there is no true underlying change in the DNA[82]. Epigenetic silencing of tumor suppressor genes is one of the most significant contributors to cancer development. Recent summaries by Esteller provide an excellent medical update of the broad reach of epigenetics in clinical medicine.

As Esteller states:

Classic genetics alone cannot explain the diversity of phenotypes within a population. Nor does classic genetics explain how, despite their identical DNA sequences, monozygotic twins or cloned animals can have different phenotypes and different susceptibilities to a disease. The concept of epigenetics offers a partial explanation of these phenomena. First introduced by C.H. Waddington in 1939 to name "the causal interactions between genes and their products, which bring the phenotype into being" epigenetics was later defined as heritable changes in gene expression that are not due to any alteration in the DNA sequence.

The best-known epigenetic marker is DNA methylation. The initial finding of global hypomethylation of DNA in human tumors was soon followed by the identification of hypermethylated tumor-suppressor genes, and then, more recently, the discovery of inactivation of microRNA (miRNA) genes by DNA methylation.

[82] See Pali and Robertson (2007)

These and other demonstrations of how epigenetic changes can modify gene expression have led to human epigenome projects and epigenetic therapies. Moreover, we now know that DNA methylation occurs in a complex chromatin network and is influenced by the modifications in histone structure that are commonly disrupted in cancer cells.

We look at three epigenetic factors; micro RNAs, Methylation, and repressor and activator genes which are not in the path. We discuss briefly the first two elements here and then we look at the third when we assemble the model.

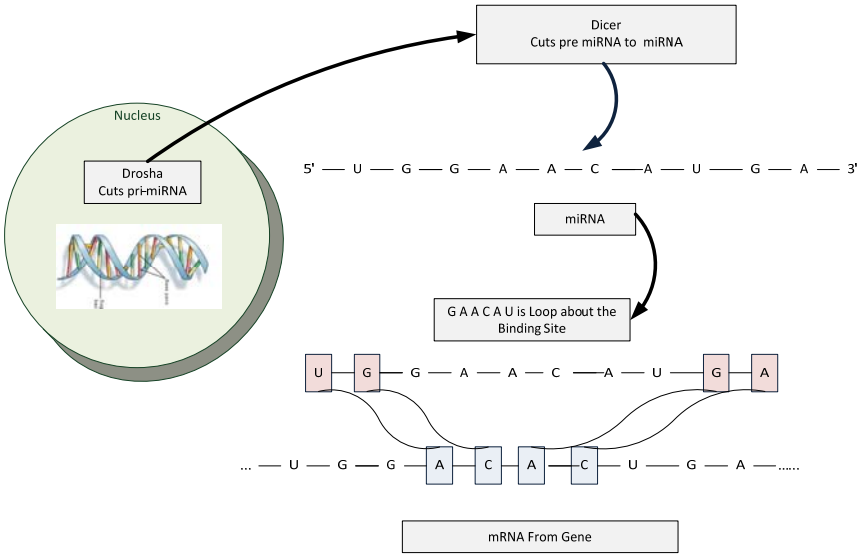
5.7.1 miRNA

This section discusses the micro RNA process and its impact on PCa. Micro RNAs, miRNA, are small single stranded RNAs which when in the cytoplasm may often bind to other RNA on complement binding sites and thus change or incapacitate the mRNA to which it binds from being translated into a protein. Craig Mello was awarded the Nobel Prize in 2006 for the discovery and his Nobel Lecture provides an excellent overview of the early stages of miRNA investigation.

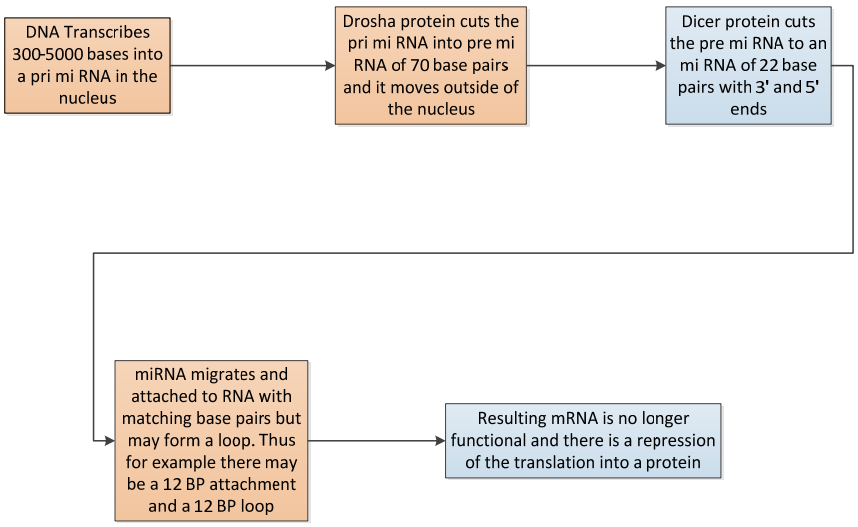
We now briefly examine the miRNA production and action. This is graphically demonstrated below. From segments of the DNA, segments not containing genes, a long segment called a pri-miRNA is generated and it is then cut to a shorter segment called a pre-miRNA and transported to the cytoplasm outside of the nucleus. Then another protein called Dicer cuts up the pre miRNA into about 22 base single-stranded pair segments which are the miRNA,

Then as we show below the small miRNA can bind to mRNA at complement sites, and in fact the binding may allow for a loop which extends out from the binding sites composed of non-complement base pairs. This binding then inactivates the mRNA and prevents its translation to a protein.

The process continues as follows (See Garcia and Miska in Appasani):



The process is described in some detail below. Here we describe the steps one at a time as is currently understood (an alternative view of this is in the paper by He and Hannon, 2004).



It is also possible for the miRNA to target more than one mRNA since the miRNA may bind in its complement binding with many other such sites on other mRNAs. It is currently not clear what the affinity of binding is for an miRNA and any possible mRNA.

Also miRNA may be obtained from introns as well as exons. The former is called intronic and the latter called exonic. Now the exonic miRNA goes through the pri and preprocess whereas the intronic miRNA is cut directly to a pre miRNA segment (see Ying et al in Appasani).

miRNAs have been identified and currently there are well over 1,000. They are named in a simple numerical order such as miRNA 34.

miRNA is a single stranded product of the process above. An alternative double stranded product is called small-interfering RNA or siRNA. siRNA usually trigger mRNA degradation whereas miRNA may cause degradation or suppression of translation to proteins. For this section we shall not focus a great deal on the siRNA functions.

Now there may be some dynamics associated with this miRNA process as well. The model above assumes a simple one to one matching of miRNA and mRNA. However the generation of the two RNAs can be continuous and we should be looking at the concentrations. Thus if we define:

[miRNA] to be the concentration of the miRNA

and

[mRNA] the concentration of the targeted mRNA

then we have a dynamic process. Namely we can see a process such as follows:

If [miRNA] < [mRNA] then there will be excess mRNA and its product protein P will have a [P] >0. Otherwise the miRNA will bind to all mRNA and there will be no resultant protein.

One may view miRNA as a buffer agent which controls the [P] of its associated [mRNA]. One can see in dynamic form the following model:

$$\frac{d[miRNA_i]}{dt} = K_{mi,i}[Pro_{mi,i}]$$

and

$$\frac{d[mRNA_i]}{dt} = K_{m,i}[Pro_{m,i}] - \kappa_i[miRNA_i]$$

where

$$[Pro] = Concentration\ of\ related\ promoter$$

Now since the binding is not necessarily 1:1, namely the miRNA may bind to several mRNA, then we may want to expand the above as follows:

$$\frac{d[miRNA_i]}{dt} = K_{mi,i}[Pro_{mi,i}]$$

and

$$\frac{d[mRNA_i]}{dt} = K_{m,i}[Pro_{m,i}] - \sum_{n=1}^{N} \kappa_{i,n}[miRNA_{i,n}]$$

where

$[Pro]$ = *Concentration of related promoter*

5.7.2 miRNA and Cancer

There has been a great amount of research regarding the impact of miRNA on cancer and especially on PCa. miRNAs may downregulate tumor suppressor genes such as PTEN. This has been seen in miRNA 21. Colin and Croce have provided several review article regarding miRNA and their influence on cancers. They argue that miRNA alterations are heavily involved in the initiation of many cancers. Their focus had been on CLL, chronic lymphocytic leukemia, and its initiating miRNAs, miR 15 and miR 16. Coppola et al (2010) provide a detailed summary of miRNAs and PCa.

For example miR34 can cause the activation and recapitulate p53 which in turn induces cell cycle arrest and apoptosis. Loss of the miR34 can result in the impairment of the p53 control of apoptosis and permit the cells to proliferate. Coppola et al perform a detailed analysis of all of the above related miRNAs and their resultant impact on PCa. miR-21 up-regulation leads to PTEN loss and thus is an oncogene.

Recent work by Poliseno et al has shown that PTEN can be down regulated via miR-106b. It had already been known that PTEN could be down-regulated by miR-22, miR-25 and miR-302. Their work demonstrated that miR-22 and miR-106b are overexpressed in PCa miR-106b is an intronic miRNA. The work of Poliseno thus has demonstrated a proto-oncogenic miRNA dependent network that regulates PTEN and thus can have a significant role in initiating PCa.

Micro RNAs are regulators of mRNA, the post transcriptional result which is then used to generate via translation the operative protein. Currently there are nearly 1,000 identified miRNAs. They are generally 22 nucleotides long, short segments, and they usually target specific mRNA and silence it. Each one of the miRNA may act upon many mRNAs.

As He and Hannon state:

Non-coding RNAs participate in a surprisingly diverse collection of regulatory events, ranging from copynumber control in bacteria[1] to X-chromosome inactivation in mammals[2].MicroRNAs (miRNAs) are a family of 21–25-nucleotide small RNAs that, at least for those few that have characterized targets, negatively regulate gene expression at the post-transcriptional leve.

Members of the miRNA family were initially discovered as small temporal RNAs (stRNAs) that regulate developmental transitions in Caenorhabditis elegans[6]. Over the past few years, it has

become clear that stRNAs were the prototypes of a large family of small RNAs, miRNAs that now claim hundreds of members in worms, flies, plants and mammals.

The functions of miRNAs are not limited to the regulation of developmentally timed events. Instead, they have diverse expression patterns and probably regulate many aspects of development and physiology. Although the mechanisms through which miRNAs regulate their target genes are largely unknown, the finding that at least some miRNAs feed into the RNA INTERFERENCE (RNAi) pathway has provided a starting point in our journey to understand the biological roles of miRNAs.

miRNAs are simple yet complex entities and key players in the epigenetics which control gene expression.

It is clear from the above that miRNAs can positively and negatively impact many elements in the pathways we have considered in HGPIN and PCa. Coppola et al review several of the key ones. For example:

- miR-146: Down regulates the AR.
- miR-34: Can recapitulate p53 resulting in apoptosis and arrest.
- miR-23: can result in c-myc overexpression and cell proliferation.

In a recent paper by Poliseno et al they have identified several others:

- miR-106b: Down-regulates PTEN and triggers PIN in murine models.
- miR-22, miR-25, miR-302: Down-regulating of PTEN.

Similarly the papers by Petrocca et al and that by Calin and Croce detail many of the miRNAs and their impacts on many cancers. As seen in the above graphic these are but a few in the overall targeting of PCa control genes. As Coppola et al state:

The hypothesis that miRs can be regarded as new broad-spectrum oncogenes or tumor suppressor genes has opened a revolutionary field of research with exciting diagnostic and therapeutic perspectives.

The compelling hint of a widespread miR deregulation in cancer pathogenesis came from the analysis of the genomic distribution of 186 miR. In this study, it was demonstrated that more than half of them mapped in cancer-associated genomic regions, namely in chromosomal sites prone to deletions, amplifications or recombinations. These aberrations can result in miR down- or up-regulation, conferring selective advantages to mutated cells.

Additional mechanisms of miR deregulation include altered expression of miRs as a consequence of excessive or deficient processing; aberrant transcription of the precursors by epigenetic silencing of miR promoters or as a result of the activity of oncogenic transcription factors; and more rarely, point mutations in mature miRs or in target sequences that can interfere with normal target recruitment

The problem that we will have in any modeling of HGPIN and PCa is not only do we have issues regarding the somewhat well-known genes but the impact of the epigenetic factors is unknown, complex, and possibly random.

Furthermore miRNAs can act in a positive or negative manner depending upon the cell and the activated networks in the cell. From Croce (2009) we have:

Importantly, miRNAs should not be described as oncogenes or tumor suppressor genes, unless the tissue or cell type involved in their action is specified. For example, miR-221 and miR-222 target an oncogene, KIT, and inhibit the growth of erythroblastic leukaemia[30], and therefore function as tumor suppressors in erythroblastic cells. but they also target at least four important tumor suppressors −phosphatase and tensin homologue (PTEN), p27, p57 and tissue inhibitor of metalloproteinases 3 (TIMP3) −and function as oncogenic miRNAs by suppressing these tumor suppressors in various human solid tumours[31] (TABLE 1). Therefore, before describing an miRNA as a tumor suppressor or an oncogene, it is necessary to specify in which cell or tissue, as cellular context is crucial for the function of miRNAs….

Recent work on miR-34 has demonstrated its impact on p53 (Rokhlin et al) and the fact that miR-34 significantly mediates the role of p53 in apoptosis in AR dependent PCa.

5.7.3 MiRNA and Stem Cells

As we have indicated elsewhere, the concept of the cancer stem cell has received significant attention. There has also been a great deal of work on the area of linking miRNAs and the stem cell model for PCa. In a recent work by Liu et al (2011) the authors demonstrate the nexus between miR-34a and its ability to inhibit PCa stem cells by directly repressing CD44. They observe that cancer stem cells have been observed in many solid cancers by using the fact that CD44 adheres to the cell surface. PCa stem cells with enhance clonogenic and tumor initiating and metastatic capacities are often enriched with CD44+ cell population. The work of Liu et al demonstrated that the administration of miR-34a to PCa cells inhibited PCa metastasis and inhibited PCa regeneration. This is one of the first uses of miRNA as a tumor suppressor.

In a recent paper by Xia (2008) the author states:

The key characteristics of stem cells are that they are capable of self-renewal and differentiation. The mechanisms by which stem cells maintain self-renewal and differentiation are complicated. In the past years, protein-coding genes had been broadly investigated in stem cell self-renewal and differentiation. Recent studies indicate miRNAs as one of the most abundant classes of post-transcriptional regulators proved to be crucial in a wide range of biological processes, which suggest that miRNAs may also play essential roles in stem cell self-renewal and differentiation. Disruption of Dicer function in murine ESs influences miRNA processing and greatly impairs their ability to differentiate …

Cancer stem cells (CSCs) are the cells within a tumor that possess the capacity to self-renew and to produce the heterogeneous lineages of cancer cells that comprise the tumor. CSCs can thus only be defined experimentally by their ability of self-renewal and tumor propagation.

The implementation of this approach explains the use of alternative terms in the literature, such as "tumor-initiating cells" to describe putative CSCs. ...

The identification of growth and differentiation pathways responsible for CSC proliferation and survival will help in the discovery of novel therapeutic targets. Previous studies have shown that many signal pathways may participate in regulating CSC functions, including Wnt/β-catenin, Notch, and Sonic hedgehog homolog (SHH). The canonical Wnt cascade has emerged as a critical regulator of stem cells and activation of Wnt signalling has also been associated with various cancers ...

CSC maintenance is dependent on β catenin signaling. Moreover, because Wnt/β-catenin signalling is not essential for normal epidermal homeostasis, such a mechanistic difference may thus be targeted to eliminate CSCs and consequently eradicate squamous cell carcinomas. It is therefore hypothesized that inhibition of Wnt signaling may provide an effective way to reduce the unwanted stem cell renewal which results in cancers.

Inhibition of Wnt signalling may prove to be an effective road to inhibit the uncontrolled cell renewal that drives cancer. Acting as novel and pivotal regulators of protein-encoding genes, miRNAs will have great potential in regulating CSCs' biological functions by targeting CSCs-related signal pathway molecules.

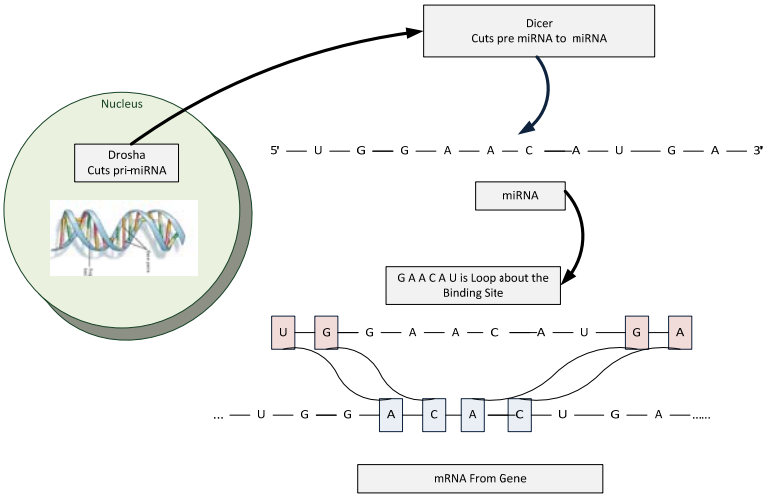
The impact of further understanding the modulating role of miRNAs and PCa will play a significant role in the development of analytic tools for PCa. The problem one all too often finds in understanding cancer models is that each step forward reveals new elements which were unknown and not readily understood. Thus incorporating the stem cell paradigm and the double edged sword of the miRNA elements will be challenging.

5.7.4 Micro RNAs and Their Functions

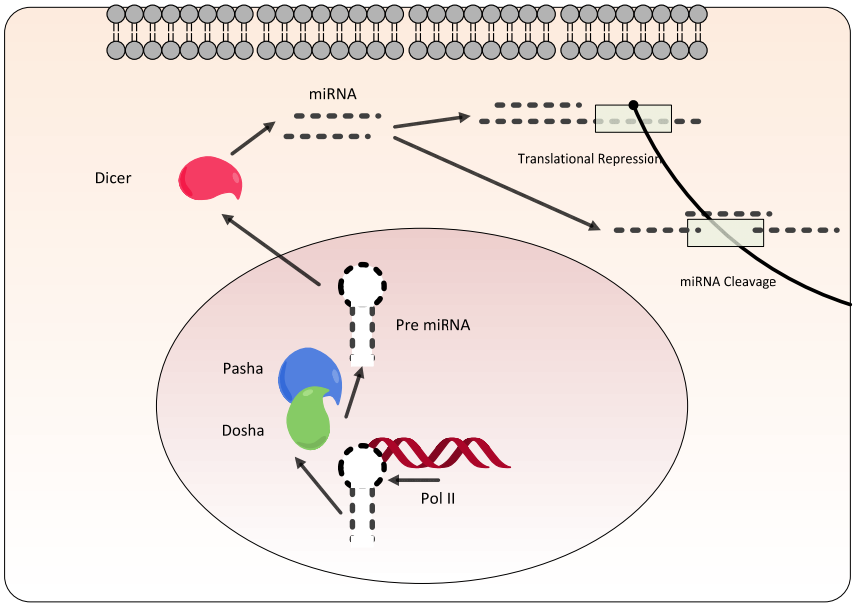
miRNAs are small (19-25 nucleotide single strand RNA) which have been created off intron sections of the DNA of a cell through pol II or pol III. They then operate on mRNA from exons which have escaped from the nucleus and are putatively maturing to proteins in the cytoplasm. Some of the proteins may be beneficial and some may not. The miRNAs seem to be secondary, and in some cases primary, pathway control elements. miRNAs contain RNA nucleotides, U, A, C, G. Thus simply stated if any possible combination is available there could be 4^{22} such miRNAs or about one trillion, equal to the national debt each year! This is a simplistic statement but it does provide a metric. We have discovered just more than a 1,000 miRNAs to data, with variants on some. Therefore a great deal more can be determined.

To demonstrate the recent occurrence of miRNA, it was not until the 6th edition of Watson's Biology of the Gene in 2008 that we see a Chapter on controlling RNAs with miRNA (See Chapter 18). In addition even some of the recent literature lends miRNAs a place as a curiosity. In fact the more they are understood the more powerful they become.

In the classic review paper by Esquela-Kerscher, A. and F, Slack, they present an excellent discussion on miRNAs. First we present the overall construct. miRNAs are produced like all RNA and then pass through the Drosha/Pasha complex and emerge from the nucleus as a double RNA with a loop. Dicer cuts the loop creating single strand short RNAs which are the miRNA.



Now from the paper we have the more detailed description where we show how miRNA can interfere with RNA translation by either inhibiting it or by slicing the RNA and in turn also inhibiting it. We depict that below.



We rely upon that here, They state:

The biogenesis of microRNAs. *MicroRNA (miRNA) genes are generally transcribed by RNA Polymerase II (Pol II) in the nucleus to form large pri-miRNA transcripts, which are capped (7MGpppG) and polyadenylated (AAAAA). These pri-miRNA transcripts are processed by the RNase III enzyme Drosha and its co-factor, Pasha, to release the ~70-nucleotide pre-miRNA precursor product. (Note that the human let-7a-1 miRNA is shown here as an example of a pre-miRNA hairpin sequence. The mature miRNA sequence is shown in red.)*

RAN–GTP and exportin 5 transport the pre-miRNA into the cytoplasm. Subsequently, another RNase III enzyme, Dicer, processes the pre-miRNA to generate a transient ~22- nucleotide miRNA:miRNA duplex.*

This duplex is then loaded into the miRNA-associated multiprotein RNA-induced silencing complex (miRISC) (light blue), which includes the Argonaute proteins, and the mature single-stranded miRNA (red) is preferentially retained in this complex. The mature miRNA then binds to complementary sites in the mRNA target to negatively regulate gene expression in one of two ways that depend on the degree of complementarity between the miRNA and its target. miRNAs that bind to mRNA targets with imperfect complementarity block target gene expression at the level of protein translation (lower left).

However, recent evidence indicates that miRNAs might also affect mRNA stability (not shown). Complementary sites for miRNAs using this mechanism are generally found in the 32 untranslated regions (3' UTRs) of the target mRNA genes. miRNAs that bind to their mRNA targets with perfect (or nearly perfect) complementarity induce target-mRNA cleavage (lower right). miRNAs using this mechanism bind to miRNA complementary sites that are generally found in the coding sequence or open reading frame (ORF) of the mRNA target.

They further detail it as follows:

MicroRNAs can function as tumour suppressors and oncogenes.

a. In normal tissues, proper microRNA (miRNA) transcription, processing and binding to complementary sequences on the target mRNA results in the repression of target-gene expression through a block in protein translation or altered mRNA stability. The overall result is normal rates of cellular growth, proliferation, differentiation and cell death.

b. The reduction or deletion of a miRNA that functions as a tumour suppressor leads to tumour formation.

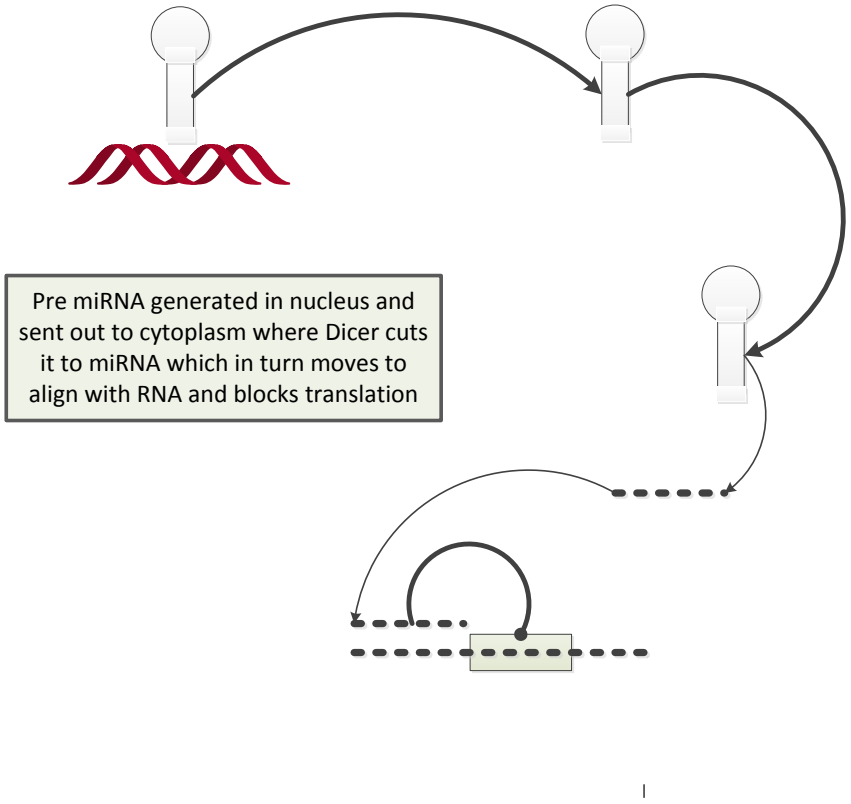
A reduction in or elimination of mature miRNA levels can occur because of defects at any stage of miRNA biogenesis (indicated by question marks) and ultimately leads to the inappropriate expression of the miRNA-target oncoprotein (purple squares). The overall outcome might involve increased proliferation, invasiveness or angiogenesis, decreased levels of apoptosis, or undifferentiated or de-differentiated tissue, ultimately leading to tumour formation.

The amplification or overexpression of a miRNA that has an oncogenic role would also result in tumour formation. In this situation, increased amounts of a miRNA, which might be produced at

inappropriate times or in the wrong tissues, would eliminate the expression of a miRNA-target tumour-suppressor gene (pink) and lead to cancer progression.

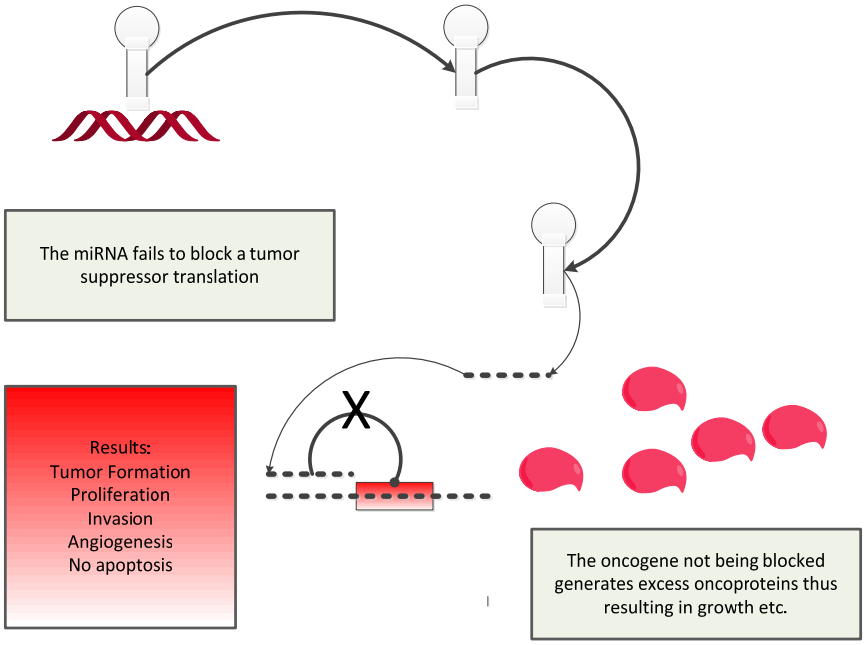
Increased levels of mature miRNA might occur because of amplification of the miRNA gene, a constitutively active promoter, increased efficiency in miRNA processing or increased stability of the miRNA (indicated by question marks). ORF, open reading frame.

We depict these three cases shown as follows. First, miRNA acting in a normal manner. This is below:

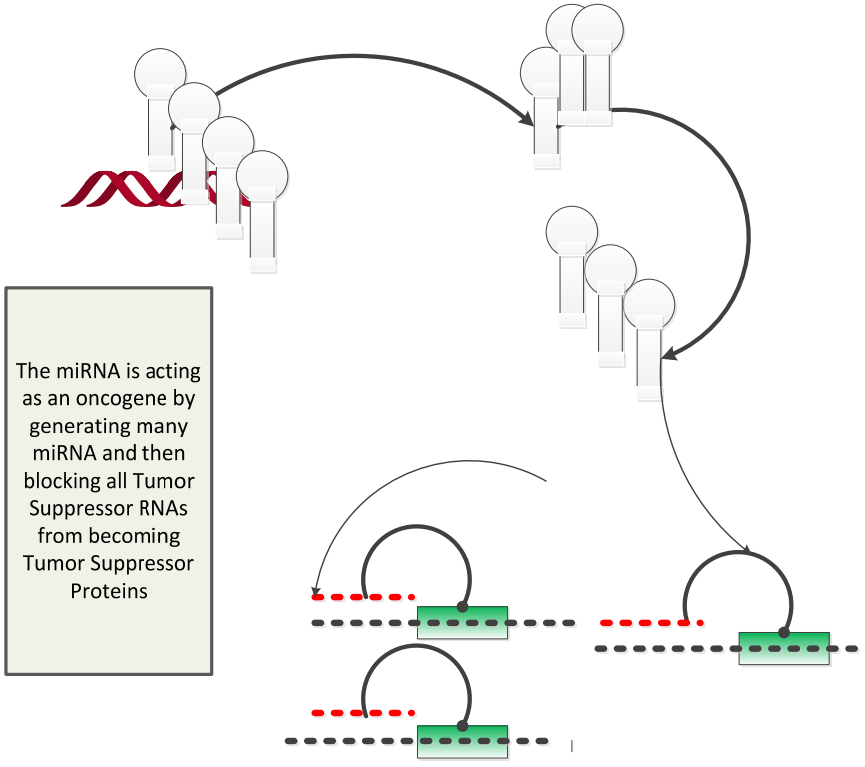


Notice above the miRNA is assumed to be a normal part of the control mechanism of the control of the conversion of the mRNA into a protein. It blocks the conversion.

Second, we now consider the second case. Here we have an oncogene which is not blocked by the miRNA and it results in many oncoproteins as shown below.



Third and finally in case 2 we have a massive explosion of miRNAs acting as onco activators as shown below.



These methods demonstrate in a somewhat simple manner how the miRNA functions in the case of certain cancers. It also demonstrates how the miRNA can become a target for therapeutics.

5.7.5 miRNA and Melanoma

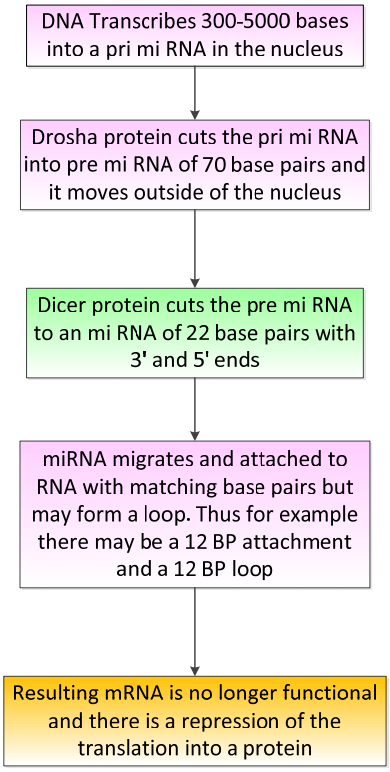
We have previously examined the impact of miRNAs in the development of cancers from several perspectives. In this brief analysis we take a recent finding regarding melanoma and a specific miRNA and then use it as a baseline to examine miRNAs in a broader context, focusing specifically on melanoma. The interest here is twofold; first, as a potential therapeutic target and second as a potential prognostic marker.

miRNAs have been examined for the past twenty years but just the last decade have they been understood specifically as elements in cancer control. Even more so, only in the past five years has their full impact been understood and the ability to manipulate certain miRNA paths controlled.

This section details many of the elements of miRNA as regards to cancer and metastatic control as well as the therapeutic control via miRNAs. What is of most significant interest is that miRNAs have such a pervasive set of control paths via activating oncogenes and suppressing genes which control metastatic growth. The miRNAs are not just control elements in select paths but appear to be control elements in the day to day paths of cellular homeostasis. This makes modeling of pathways significantly more complex.

It is critical to understand that as we have seen genomic models built around proteins, genes and pathways, we have also not seen the clear presence of miRNAs as integral parts of this process. One need just look at the many papers on pathway dynamics and almost to each one there is a total absence of miRNAs. We had proposed about five years ago that we look at miRNAs as noise, as at best epigenetic accidents which result in loss of expression. Now however it may be argued that they play as significant a role as the well-known pathways, albeit not yet fully understood.

Let us recall that the miRNA functions in a manner shown below:



We shall detail this process later in the document. However it is good to understand the nature of the miRNA. One key factor is that reproducing and introducing miRNAs appears to be rather straightforward. This perhaps they represent a powerful tool in the therapeutic arsenal.

The specific focus here is on miRNA-26a[83]. There are many databases now with a great deal of information regarding the miRNAs and we refer to them as in course.

5.7.5.1 Recent Observations

We begin by examining a recent paper regarding miR-26a. As we shall discuss later this miRNA is found to be aberrant in multiple cancers and in the case of melanoma the disruption associated with several pathways is somewhat clearly understood. In a recent paper by Reuland et al the authors make the following observations[84]:

Melanoma is an aggressive cancer that metastasizes rapidly and is refractory to conventional chemotherapies. Identifying microRNAs (miRNAs) that are responsible for this pathogenesis is therefore a promising means of developing new therapies. We identified miR-26a through microarray and quantitative reverse-transcription–PCR (qRT-PCR) experiments as a miRNA that is strongly downregulated in melanoma cell lines as compared with primary melanocytes. Treatment of cell lines with miR-26a mimic caused significant and rapid cell death compared with a negative control in most melanoma cell lines tested.

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[83] http://www.mirbase.org/cgi-bin/mirna_entry.pl?acc=MI0000083

[84] http://www.nature.com/jid/journal/vaop/ncurrent/full/jid2012400a.html

In surveying targets of miR-26a, we found that protein levels of SMAD1 (mothers against decapentaplegic homolog 1) and BAG-4/SODD were strongly decreased in sensitive cells treated with miR-26a mimic as compared with the control.

The luciferase reporter assays further demonstrated that miR-26a can repress gene expression through the binding site in the 3' untranslated region (3'UTR) of SODD (silencer of death domains). Knockdown of these proteins with small interfering RNA (siRNA) showed that SODD has an important role in protecting melanoma cells from apoptosis in most cell lines sensitive to miR-26a, whereas SMAD1 may have a minor role. Furthermore, transfecting cells with a miR-26a inhibitor increased SODD expression. Our findings indicate that miR-26a replacement is a potential therapeutic strategy for metastatic melanoma, and that SODD, in particular, is a potentially useful therapeutic target.

The observations focus on several key areas:

1. The impact of miRNAs on melanoma metastasis. As we will discuss there have been many previous studies implicating many miRNAs in this area. Thus seems to expand the results.

2. There appears to be a therapeutic approach to the issue by increasing the miRNA26a to further reduce by binding to the SODD facilitator product. There again have been several studies along this line recently. SODD is an interesting controlling gene/protein complex and the control via miR-26a is of significance.

3. There may be a prognostic indicator here as well. Again there has been a great deal of work in this field.

First we examine both the miRNA26a and SODD respectively and then we examine the issues discussed above in some detail. This represents just another of many studies regarding the use of miRNAs for the potential control of melanoma.

Before continuing it is useful to examine some of the additional comments the authors of the referred to article have made to the trade press relating to the release of the paper. Now one trade press article states[85]:

A University of Colorado Cancer Center study in this month's edition of the Journal of Investigative Dermatology describes a new target and potential treatment for melanoma, the most dangerous form of skin cancer. MicroRNA can decide which genes in a cell's DNA are expressed and which stay silent. Melanoma tends to lack microRNA-26a, which makes the gene SODD go silent.

"It's a double negative," says Yiqun Shellman, PhD, investigator at the CU Cancer Center, associate professor at the CU School of Medicine, and the study's co-senior author. "miR-26a

85 http://medicalxpress.com/news/2012-12-serendipity-potential-therapy-melanoma.html also http://medicalxpress.com/news/2012-12-serendipity-potential-therapy-melanoma.html#jCp

works to stop the growth of cancer. You turn off this thing that should stop growth, and you have growth." When Shellman, David Norris and colleagues reintroduced microRNA-26a to melanoma cell lines that lacked it, they saw a marked decrease in cancer cell survival. MicroRNA-26a killed melanoma cells while leaving healthy cells unharmed. In fact, the discovery started back a couple steps.

First the group compared microRNA expression in healthy cells to that of microRNA expression in melanoma cells. "We hoped the difference between microRNA expression in healthy and melanoma cells would show which ones were contributing to tumorgenesis," Shellman says.

The microRNA most consistently different between healthy and cancerous cells was 26a. The discovery of how it works and what exactly it does was serendipitous. "We started by testing the effect of microRNA-26a on known gene targets to see if it was effecting the expression of logical, cancer-causing pathways, but none of them seemed affected in melanoma," Shellman says.

"We were working with the SODD gene in an unrelated project, and SODD has a putative but not high-scored binding site for miR-26a, and thought, why not test it? Sure enough, it turned out to be the target – microRNA-26a downregulates this gene." Shellman hopes this robust finding in cell cultures will help pave the way for future work with microRNA-26a as a therapeutic target in animal models and eventually a human trial.

"The first step is to further pinpoint the genetic signatures of the patients likely to benefit from microRNA-26a replacement therapy," Shellman says, noting that only some and not all melanoma cells were killed by miRNA replacement. "Maybe it's simply the downregulation of microRNA-26a itself, or maybe we can use SODD expression as the biomarker," Shellman says.

Once Shellman and colleagues discover the characteristics of a melanoma susceptible to microRNA-26a treatment, they hope funding will allow the lab to follow the promising therapy up the evolution from cells to humans.

As can be seen from the conversation above, there still may exist some questions of the details of the process. What is critical, however, is the fact that the miRNA plays such a prominent role, that one may target the miRNA, and that a pathway is a fundamental part of the development of a putative therapeutic. But fundamentally the last sentence above does diminish the ultimate enthusiasm.

The critical observations made here is the relationship between the controlling proteins, their related mRNA and the interference coming from miRNA. This has not been explored in significant detail until of late.

Another trade press review states as follows[86]:

[86] http://www.redorbit.com/news/health/1112752907/genetic-culprit-for-melanoma-found-122112/

Researchers from the University of Colorado Cancer Center say that they have discovered a new, more targeted way of treating melanoma, the most deadly form of skin cancer.
The findings, described in a recent edition of the Journal of Investigative Dermatology, describe how small pieces of genetic material known as MicroRNA can choose the genes in a DNA cell that are either expressed or kept silent. With melanoma in particular, the researchers discovered a deficiency of microRNA-26a that usually silences the gene SODD.

"It's a double negative," explained the study's co-senior author Yiqun Shellman, an investigator at the University of Colorado Cancer Center and associate professor at the University of Colorado School of Medicine, in a prepared statement. "MiR-26a works to stop the growth of cancer. You turn off this thing that should stop growth, and you have growth."

In the study, melanoma cell lines that lacked microRNA-26a were reintroduced to the cell in a lab. As a result, there was a reduction in cancer cell survival and the microRNA-26a eliminated melanoma cells while leaving healthy cells alive. The team of investigators was able to compare the expression of microRNA in healthy cells to the expression of microRNA in melanoma cells.

"We hoped the difference between microRNA expression in healthy and melanoma cells would show which ones were contributing to tumorgenesis," continued Shellman in the statement.
The researchers saw that the expression of micro-RNA-26 was consistently different between healthy and cancerous cells. Some, but not all, of the melanoma cells were eliminated by the replacement introduction of mRNA.

"The first step is to further pinpoint the genetic signatures of the patients likely to benefit from microRNA-26a replacement therapy," noted Shellman in the statement. "Maybe it's simply the downregulation of microRNA-26a itself, or maybe we can use SODD expression as the biomarker."

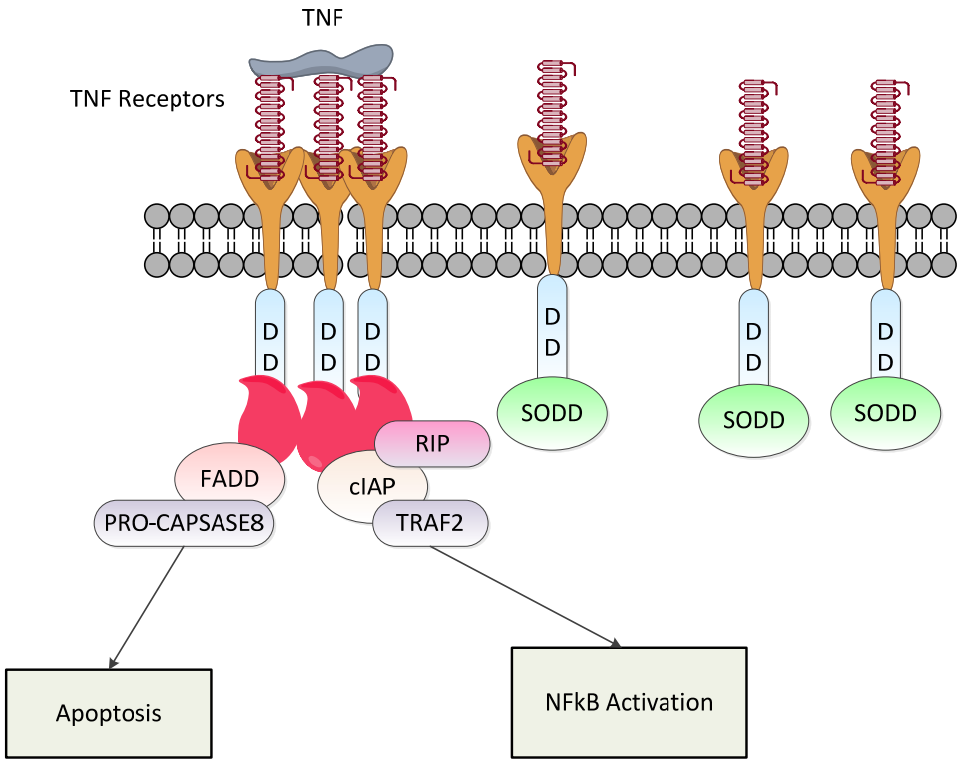
Moving forward, Shellman believes that her team's discovery of the role of MicroRNA in the development of carcinoma in cell cultures may eventually help develop new therapeutic techniques that could be used in real cancer patients.

This above statement is a simple reiteration of some of the prior work. Again it is clear that although experimentally observed, one is still quite a way from clinical reality.

5.7.5.2 SODD Pathway

Let us first consider the SODD pathway. From the Biocarta database we have the following graphic with the text noted below[87].

[87] http://www.biocarta.com/pathfiles/h_soddpathway.asp



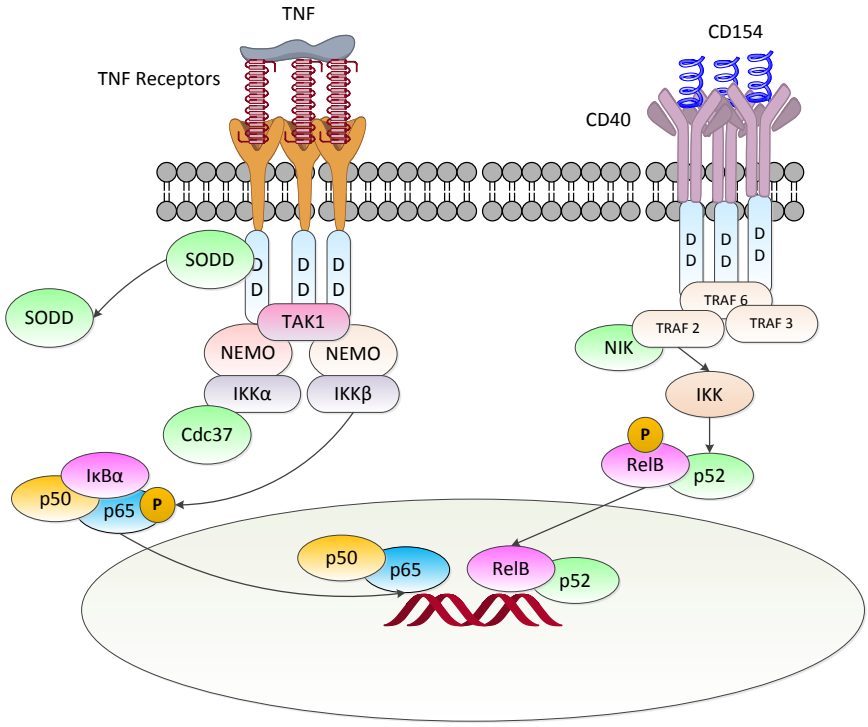
The tumor necrosis factor (TNF) receptor superfamily contains several members with homologous cytoplasmic domains known as death domains (DD). The intracellular DD are important in initiating apoptosis and other signaling pathways following ligand binding by the receptors. In the absence of ligand, DD-containing receptors are maintained in an inactive state.

TNF RI contains a cytoplasmic DD required for signaling pathways associated with apoptosis and NF-kB activation. Jiang et al. identified a widely expressed 60 kDa protein, named SODD (silencer of death domains), and associated with the DD of TNF RI and DR3. Overexpression of SODD suppresses TNF-induced cell death and NF-kB activation demonstrating its role as a negative regulatory protein for these signaling pathways.

TNF-induced receptor trimerization aggregates the DD of TNF RI and recruits the adapter protein TRADD. This in turn promotes the recruitment of the DD-containing cytoplasmic proteins FADD, TRAF2 and RIP to form an active TNF RI signaling complex (Figure 1A). In contrast, SODD acts as a silencer of TNF RI signaling and does not interact with TRADD, FADD, or RIP (Figure 1B).4 It is associated with the DD of TNF RI and maintains TNF RI in an inactive, monomeric state. TNF-induced aggregation of TNF RI promotes the disruption of the SODD-TNF RI complex.

SODD does not interact with the DD of other TNF receptor superfamily members such as Fas, DR4, DR5, or TNF RII. SODD association with TNF RI may represent a general model for the prevention of spontaneous TNF signaling by other DD-containing receptors.

In a recent paper by Viatour et al, the authors examine the specifics of the NF-kB pathway elements regarding inflammation and cancer. We take the figure below as modified from their work. It demonstrates the SODD impact as an integrated whole.



As Viatour et al state regarding the above Figure[88] which is detailed in the footnote[89]:

The classical (blue arrows), alternative (green arrows) and atypical (purple arrows) NF-κB-activating pathways as illustrated by the TNF-α-mediated, CD40-mediated and DNA-damage-mediated NF-κB activation pathways, respectively. In the classical NF-κB-activating pathway, upon binding of TNFα to TNFR1, SODD is released from the receptor and triggers the sequential recruitment of the adaptors TRADD, RIP and TRAF2 to the membrane.

Then, TRAF2 mediates the recruitment of the IKK complex – composed of IKKα, IKKβ and NEMO – to the TNFR1 signalling complex. Hsp90 and Cdc37 are also part of the IKK complex and are required for the TNFα-induced IKK activation and shuttling of the IKK complex from the cytoplasm to the membrane, and ELKS connects IκBα to the IKK complex [83].

88 http://www.sciencedirect.com/science/article/pii/S0968000404002993

89 Abbreviations: CK2, casein kinase 2; ELKS, Glu-Leu-Lys-Ser; Hsp90, heat shock protein 90; IκB, inhibitor of NF-κB; IKK, IκB kinase; NEMO, NF-κB essential modulator; NF-κB, nuclear factor-κB; NIK, NF-κB-inducing kinase; RIP, receptor-interacting protein; RSK1, ribosomal S6 kinase 1; SODD, silencer of death domains; TNF-α, tumour necrosis factor α; TNFR1, TNF receptor 1; TRADD, TNF-receptor-associated death domain protein; TRAF, TNF-receptor-associated factor; Ub, ubiquitin.

Activation of the IKK complex leads to the phosphorylation of IκBα at specific residues, ubiquitination through binding of ubiquitin proteins and degradation of this inhibitory molecule via the proteasome pathway. Then, the heterodimer p50–p65 is released and migrates to the nucleus where it binds to specific κB sites and activates a variety of NF-κB target genes, including IL-8, IL-6, TNFα and many more.

The alternative pathway is triggered by binding of the CD40 ligand to its receptor, leading to recruitment of TRAF proteins and the sequential activation of NIK and IKKα, which then induces the processing of the inhibitory protein p100. p100 proteolysis releases p52 which forms heterodimers with RelB. This pathway is NEMO-independent and relies on IKKα homodimers. The atypical pathway, which is triggered by DNA damage such as UV, relies on sequential p38 and CK2 activations, and involves phosphorylation and subsequent IκBα degradation via an IKK-independent pathway.

Subsequently, free NF-κB moves into the nucleus to activate its target genes. Note that the DNA-damaging agent doxorubicin also triggers p65 phosphorylation via a p53- and RSK1-dependent pathway (not shown). Phosphorylation of the signalling molecules in addition to NF-κB and IκB proteins are illustrated.

Tschopp et al discuss apoptosis as in which follows[90]:

Spontaneous signaling from death-domain-containing receptors can result in inappropriate cell death. An inhibitory protein has recently been identified, called silencer of death domains (SODD), that binds to the death domain of tumor necrosis factor receptor 1, thereby negatively regulating downstream signaling.

Members of the tumor necrosis factor (TNF) ligand family are critically involved in the regulation of infections, inflammation, autoimmune diseases and tissue homeostasis. Binding of these ligands to their respective receptors leads to the triggering of diverse signaling pathways, including the activation of caspases, the activation and nuclear translocation of nuclear factor κB (NF-κB), and the activation of mitogen-activated protein kinases such as Jun N-terminal kinase (JNK). As each of these pathways leads to different outcomes, TNF-related ligands can promote apoptosis, differentiation, or proliferation.

The TNF-related factors are synthesized as trimeric type II transmembrane molecules that are capable of oligomerizing (trimerizing) the corresponding receptor. Consequently, ligand-bound receptors transmit the signal across the membrane by bringing their cytoplasmic portions into close proximity, leading to the recruitment and activation of downstream effector proteins. Those members of the TNF receptor family that induce apoptosis have in their cytoplasmic region a sequence of approximately 70 amino acids called the death domain, which is essential for signal transmission.

These domains represent protein–protein interaction motifs that have an intrinsic propensity to oligomerize and to interact with other death domains in a homophilic fashion. At present, six

[90] http://www.sciencedirect.com/science/article/pii/S0960982299802334

death-domain-containing receptors have been identified — Fas, TNF receptor 1 (TNF-R1), death receptor 3 (DR3; also known as TRAMP, Wsl, APO-3 and LARD), the two receptors for TNF-related apoptosis-inducing ligand (TRAIL) TRAIL-R1/DR4 and TRAIL-R2/DR5, and DR6

They previous figure has demonstrated the processes referred to above. Now from NCBI we have the definition and description of SODD (SODD (silencer of death domains) ; or BAG-4 8p11.23, BAG4 BCL2-associated athanogene) as follows[91]:

The protein encoded by this gene is a member of the BAG1-related protein family. BAG1 is an anti-apoptotic protein that functions through interactions with a variety of cell apoptosis and growth related proteins including BCL-2, Raf-protein kinase, steroid hormone receptors, growth factor receptors and members of the heat shock protein 70 kDa family.

This protein contains a BAG domain near the C-terminus, which could bind and inhibit the chaperone activity of Hsc70/Hsp70. This protein was found to be associated with the death domain of tumor necrosis factor receptor type 1 (TNF-R1) and death receptor-3 (DR3), and thereby negatively regulates downstream cell death signaling.

The regulatory role of this protein in cell death was demonstrated in epithelial cells which undergo apoptosis while integrin mediated matrix contacts are lost. Alternatively spliced transcript variants encoding distinct isoforms have been identified

The critical observation is the role of SODD in apoptosis. If it is inhibited then cell death is prevented.

Similarly for SMAD, NCBI states[92] (SMAD1 SMAD family member 1 or BSP1; JV41; BSP-1; JV4-1; MADH1; MADR1 at 4q31):

The protein encoded by this gene belongs to the SMAD, a family of proteins similar to the gene products of the Drosophila gene 'mothers against decapentaplegic' (Mad) and the C. elegans gene Sma. SMAD proteins are signal transducers and transcriptional modulators that mediate multiple signaling pathways. This protein mediates the signals of the bone morphogenetic proteins (BMPs), which are involved in a range of biological activities including cell growth, apoptosis, morphogenesis, development and immune responses.

In response to BMP ligands, this protein can be phosphorylated and activated by the BMP receptor kinase. The phosphorylated form of this protein forms a complex with SMAD4, which is important for its function in the transcription regulation. This protein is a target for SMAD-specific E3 ubiquitin ligases, such as SMURF1 and SMURF2, and undergoes ubiquitination and proteasome-mediated degradation. Alternatively spliced transcript variants encoding the same protein have been observed.

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91 http://www.ncbi.nlm.nih.gov/gene/9530

92 http://www.ncbi.nlm.nih.gov/gene/4086

5.7.6 miRNA-26a in Other Cancers

miRNA miR-26a has been found to play a role in other cancers. We examine a few of these malignancies here based upon work in the literature.

5.7.6.1 Lung Cancer

Dang et al state regarding miR-26a the following[93]:

MicroRNAs (miRNAs) are a class of 21-23 nucleotide RNA molecules that play critical roles in the regulation of various cancers, including human lung cancer.

Among them, miR-26a has been identified as a tumor-related regulator in several cancers, but its pathophysiologic properties and correlation with the development of human lung cancer remain unclear. In this study, it was determined that miR-26a expression is clearly down-regulated in human lung cancer tissues relative to normal tissues.

Meanwhile, the overexpression of miR-26a in the A549 human lung cancer cell line dramatically inhibited cell proliferation, blocked G1/S phase transition, induced apoptosis, and inhibited cell metastasis and invasion in vitro. In contrast, a miR-26a inhibitor was used to transfect A549 cells, and the inhibition of endogenous miR-26a promoted cell metastasis and invasion. In addition, miR-26a expression inhibited the expression of enhancer of zeste homolog 2 (EZH2) and transactivated downstream target genes, including disabled homolog 2 (Drosophila) interacting protein gene (DAB2IP) and human Runt-related transcription factor 3 (RUNX3), which suggests that EZH2 is a potential target of miR-26a as previously reported. In conclusion, miR-26a plays an important role as an anti-oncogene in the molecular mechanism of human lung cancer and could potentially be used for the treatment of lung cancer.

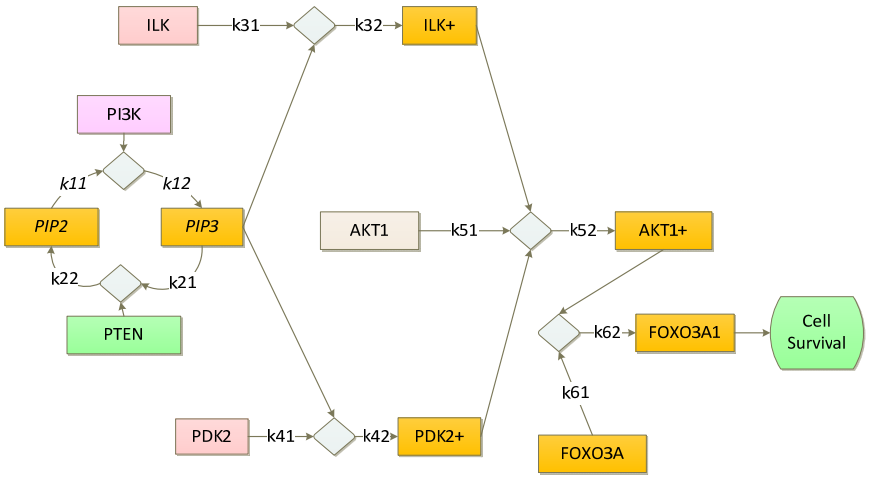
5.7.6.2 Glioma

Huse et al have examined miRNA26a in glimoas. Specifically they state:

Activated oncogenic signaling is central to the development of nearly all forms of cancer, including the most common class of primary brain tumor, glioma. Research over the last two decades has revealed the particular importance of the Akt pathway, and its molecular antagonist PTEN (phosphatase and tensin homolog), in the process of gliomagenesis.

Before continuing we present part of the AKT pathway as related to cell survival as below:

[93] http://www.ncbi.nlm.nih.gov/pubmed/22469510



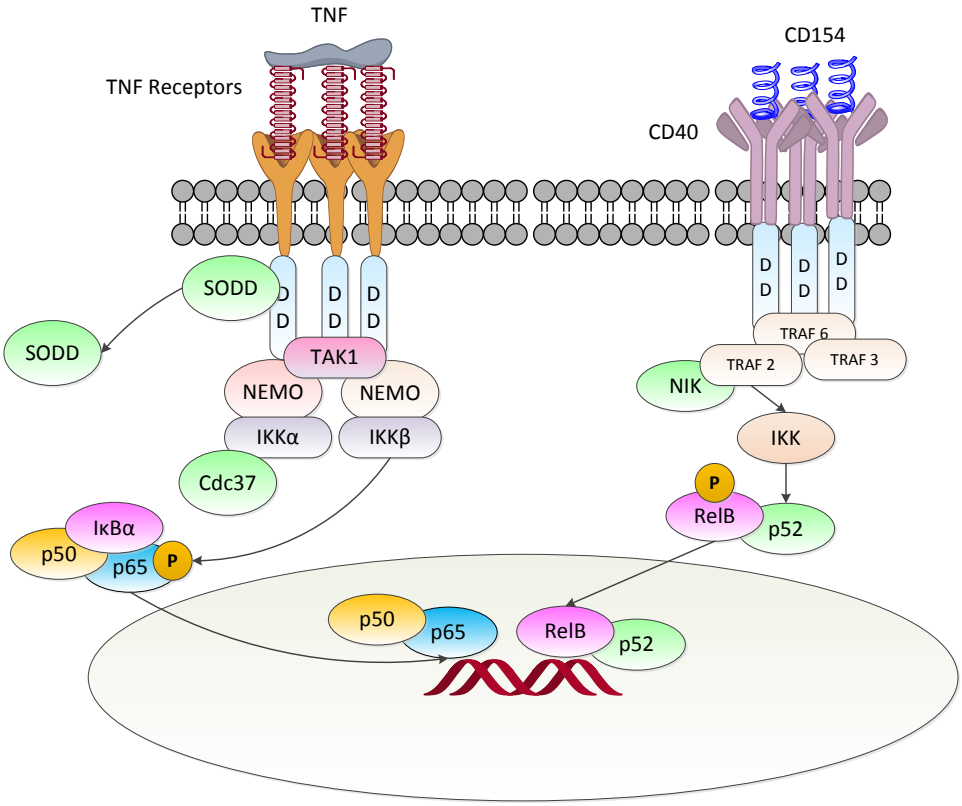
Note in this above graphic we show putative reaction rates associated with AKT and its up and down stream control elements. We have not depicted any miRNA control elements. The reason for this is that when this was developed there was no full recognition for how miRNA did interfere. We continue with the article on gliomas as follows:

Recent studies have also demonstrated that microRNAs (miRNAs) may be responsible for the modulation of cancer-implicated genes in tumors. Here we report the identification miR-26a as a direct regulator of PTEN expression. We also show that miR-26a is frequently amplified at the DNA level in human glioma, most often in association with monoallelic PTEN loss.

Finally, we demonstrate that miR-26a-mediated PTEN repression in a murine glioma model both enhance de novo tumor formation and preclude loss of heterozygosity and the PTEN locus. Our results document a new epigenetic mechanism for PTEN regulation in glioma and further highlight dysregulation of Akt signaling as crucial to the development of these tumors.

Chario presents the following graphics, as we have modified and simplified it, to describe the process[94]:

[94] http://www.sciencedirect.com/science/article/pii/S0962892409001342

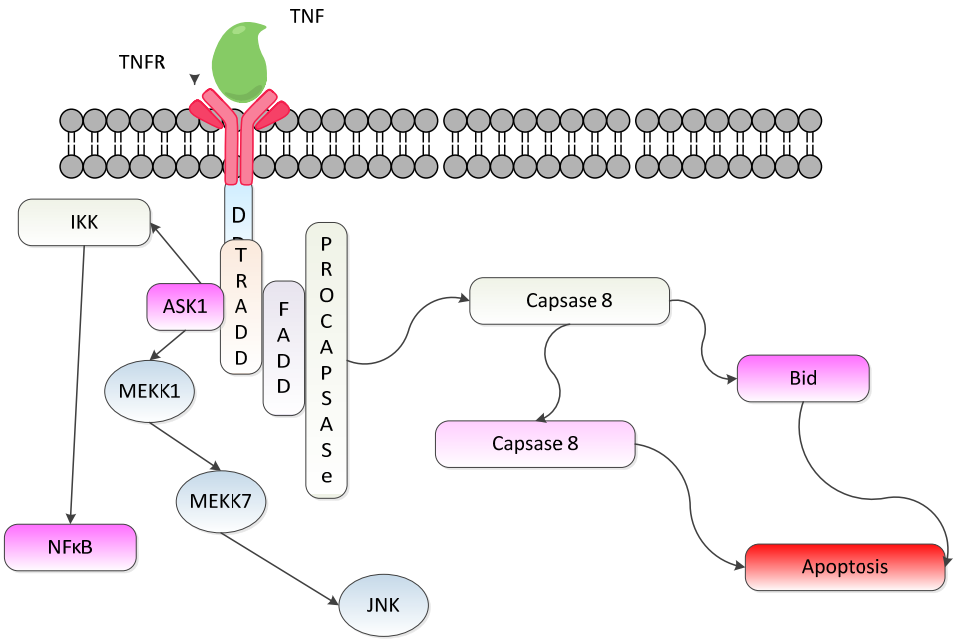


Chario then discusses some details of these pathways. We have examined them in detail before. He starts by stating

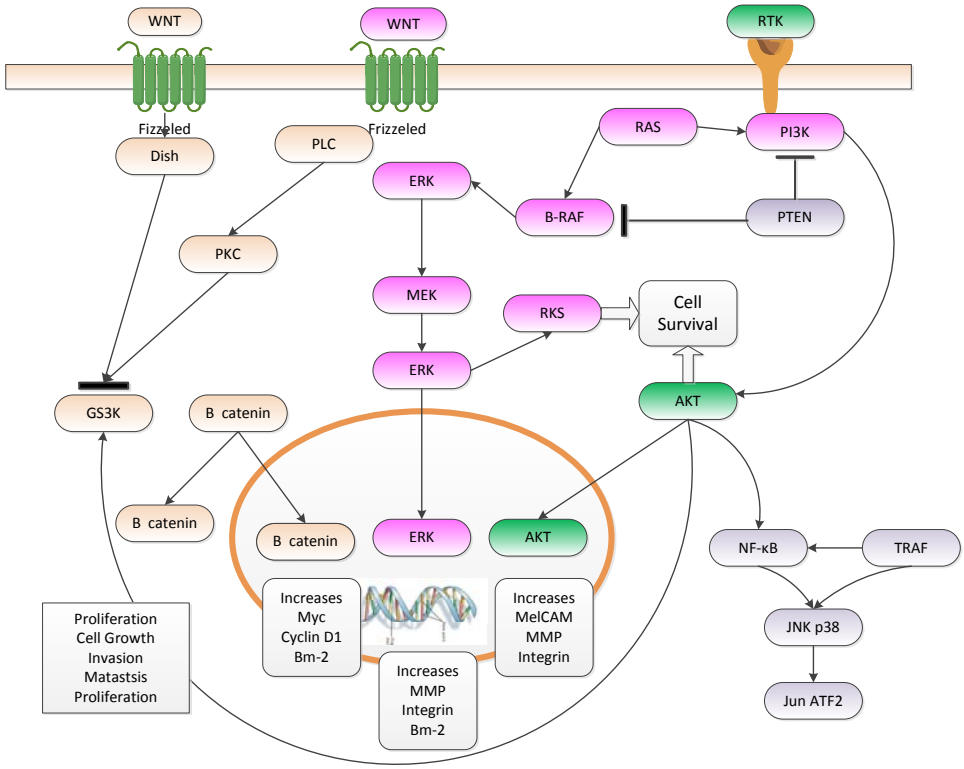
The IκB kinase (IKK) complex is involved in transcriptional activation by phosphorylating the inhibitory molecule IκBα, a modification that triggers its subsequent degradation, enabling activation of nuclear factor kappa B (NF-κB). Importantly, recent reports indicate that multiple cytoplasmic and nuclear proteins distinct from the NF-κB and IκB proteins are phosphorylated by the catalytic subunits of the IKK complex, IKKα or IKKβ.

Here, I describe how IKK subunits can have crucial roles in allergy, inflammation and immunity by targeting proteins such as SNAP23 and IRF7, but also in cancer by phosphorylating key molecules such as p53, TSC1 and FOXO3a through NF-κB-independent pathways. Thus, these recent findings considerably widen the biological roles of these kinases and suggest that a full understanding of the biological roles of IKKα and IKKβ requires an exhaustive characterization of their substrates.

Now Chario considers several cases in detail. The graphic above sets the general stage. He considers both immune and cancerous effects on the NF-κB functions. First recall the NF-κB production as shown below (see Barrett):



A more detailed view is shown below:



We can see the many well accepted pathways being processed.

Now Chario relates three IKK pathway details. We use the above diagrams as points of reference.

5.7.6.2.1 The IKKα- and IKKβ-dependent NF-κB-activating pathways.

On the left is the TNFα-dependent signalling pathway. Upon binding of TNFα to the TNFR1, SODD is released from the receptor and triggers the sequential recruitment of the adaptors TRADD, RIP and TRAF2 to the membrane. Then, TRAF2 mediates the recruitment of the IKK complex, composed of IKKα, IKKβ and NEMO, to the TNFR1 signalling complex. The scaffold proteins TAB2 and TAB3 subsequently bind to Lys63-polyubiquitylated substrates, such as RIP1, resulting in TAK1 and then IKKβ activations.

Whereas the receptor-proximal events that include the recruitment and activation of TAK1 rely on Lys63-linked polyubiquitylations, linear ubiquitin (Ub) chains are preferentially sensed by the UBAN motif of NEMO (not illustrated, see text for details). Hsp90 and Cdc37 are also part of the IKK complex and are required for the TNFα-induced IKK activation and shuttling of the IKK complex from the cytoplasm to the membrane. Activation of IKKβ leads to IκBα phosphorylation on specific residues, polyubiquitylation through binding of ubiquitin proteins and its degradation through the proteasome pathway.

Then, the heretodimer p50–p65 binds to specific κB sites and activates a variety of NF-κB target genes coding for pro-inflammatory cytokines (IL-6) and chemokines. A variety of proteins including TAK1, IKKβ, NEMO and p65 are also phosphorylated upon TNFα stimulation.

On the right is the alternative NF-κB-activating pathway. Binding of CD154 triggers the classical NEMO-dependent pathway (not illustrated) and the NEMO-independent cascade. This pathway relies on the recruitment of TRAF6 and the heterodimer TRAF2–TRAF3 to the CD40 receptor. NIK is subsequently activated by phosphorylation and polyubiquitylated in a degradative manner by the E3 ligase TRAF3. IKKα homodimers are activated by NIK and phosphorylate the inhibitory molecule p100, the partial processing of which generates the NF-κB protein p52. This latter transcription factor moves into the nucleus as heterodimer with RelB to regulate the expression of genes involved in lymphoid orgagenesis or coding for chemokines (BLC) or cytokines (BAFF).

5.7.6.2.2 The IKKα- and NF-κB-independent pathways.

Activation of the TLR9-dependent pathway triggers IKKα-mediated IRF7 phosphorylation in the cytoplasm and ultimately leads to IFN-α production. Nuclear IKKα phosphorylates the co-repressor SMRT, which triggers its nuclear export with HDAC3 and its degradation.

As a result, co-activators such as CBP are recruited and the expression of NF-κB-dependent genes is induced. CBP is also phosphorylated by IKKα in the nucleus and this modification enhances its binding to NF-κB proteins and limits the expression of the p53-dependent target genes. Upon estrogen treatment (not represented), the co-activator SRC-3 is also phosphorylated by IKKα, a modification that is required for the expression of hormone-responsive genes (cyclin D1, c-myc) and, consequently, for breast cancer cell proliferation.

5.7.6.2.3 The IKKβ- and NF-κB-independent pathways and their relevance in cancer.

TRAF2 is targeted by the deubiquitine ligase CYLD upon stimulation by TNFα and this post-translational modification facilitates the non degradative (Lys63-linked) polyubiquitylation of TRAF2. IKKβ also phosphorylates 14-3-3β upon TNFα stimulation and releases the 14-3-3β–TPP complex from ARE sequences found in multiple mRNAs.

As a result, those mRNAs coding for cytokines and chemokines are stabilized. TNFα also triggers the IKKβ-mediated phosphorylation of TSC1, which results in mTOR activation, S6K1 and 4EBP1 phosphorylations, and ultimately to the stimulation of angiogenesis through the transcriptional induction of the VEGF-encoding gene. FOXO3a is also a target of IKKβ and this phosphorylation triggers its nuclear export and its degradative polyubiquitylation in breast cancer cells harbouring constitutive IKKβ activity.

Dok1 is also phosphorylated by IKKβ upon TNFα stimulation and this modification positively regulates cell motility. IKKβ-mediated Aurora A phosphorylation causes its subsequent degradative polyubiquitylation, a pathway that is required for genome integrity.

The tumour-suppressor protein p53 is inactivated through an IKKβ-mediated phosphorylation and subsequent degradation through the proteasome pathway.

5.7.6.2.4 Hepatocellular Carcinomas

Zhu has examined miRNAs in the context of hepatocellular carcinomas. His work states that miR-26a is also present and it details other pathway control elements as well. Specifically Zhu states:

MicroRNAs (miRNAs) belong to a class of endogenously expressed, small non-coding RNAs that cause translational repression and/or mRNA destabilization by binding to the 30-untranslated regions (30-UTRs) of the target mRNAs (1). Approximately 40% of all miRNAs are located within intronic regions of protein-coding transcriptional units (TUs) (2).

Analysis of 175 human miRNAs across 24 different human organs reveals that the expression of intronic miRNAs largely coincides with the transcription of their host TUs (3), indicating that the intronic miRNAs and their host genes may be co-regulated and are generated from a common precursor transcript. Emerging evidence suggests that intronic miRNAs may be functionally associated with their host genes. Few reports suggest that there is an antagonizing effect of the intronic miRNA on the function of its host gene, as with miR-218 (4) and miR-10 (5,6).

The secreted Slit ligands and their Robo receptors constitute a Slit–Robo signaling pathway that controls the directed migration of neurons and vascular endothelial cells during embryonic development. miR-218 is localized in the intron of the Slit gene, and similar expression patterns are observed between miR-218 and Slit in different tissues.

We conducted serum starvation stimulation assays in primary fibroblasts and two-thirds partial-hepatectomies in mice, which revealed that miR-26a/b and CTDSP1/2/L were expressed concomitantly during the cell cycle process. Specifically, they were increased in quiescent cells and decreased during cell proliferation. Furthermore, both miR-26 and CTDSP family members were frequently downregulated in hepatocellular carcinoma (HCC) tissues. Gain- and loss-of-function studies showed that miR-26a/b and CTDSP1/2/L synergistically decreased the phosphorylated form of pRb (ppRb), and blocked G1/S-phase progression.

Further investigation disclosed that miR-26a/b directly suppressed the expression of CDK6 and cyclin E1, which resulted in reduced phosphorylation of pRb. Moreover, c-Myc, which is often upregulated in cancer cells, diminished the expression of both miR-26 and CTDSP family members, enhanced the ppRb level and promoted the G1/S-phase transition. Our findings highlight the functional association of miR-26a/b and their host genes and provide new insight into the regulatory network of the G1/S-phase transition.

The cyclin presence is anticipated as regards to proliferation via lost cell cycle control. The G1/S phase transition is often a major transition in malignancies.

5.7.6.3 Melanoma

Other researchers have examined miRNAs and melanoma as well. For example the work of Segura et al (2012) state:

Melanoma incidence and associated mortality continue to increase worldwide. The lack of treatments with durable responses for stage IV melanoma may be due, at least in part, to an incomplete understanding of the molecular mechanisms that regulate tumor initiation and/or progression to metastasis. Recent evidence supports miRNA dysregulation in melanoma impacting several well-known pathways such as the PI3K/AKT or RAS/MAPK pathways, but also underexplored cellular processes like protein glycosylation and immune modulation.

There is also increasing evidence that miRNA can improve patient prognostic classification over the classical staging system and provide new therapeutic opportunities. The integration of this recently acquired knowledge with known molecular alterations in protein coding genes characteristic of these tumors (i.e., BRAF and NRAS mutations, CDKN2A inactivation) is critical for a complete understanding of melanoma pathogenesis.

Here, we compile the evidence of the functional roles of miRNAs in melanomagenesis and progression, and of their clinical utility as biomarkers, prognostic tools and potential therapeutic targets. Characterization of miRNA alterations in melanoma may provide new angles for therapeutic intervention, help to decipher mechanisms of drug resistance, and improve patient classification for disease surveillance and clinical benefit.

The above work readily complements the work upon which we have focused this analysis.

Additional melanoma analyses have been done by Zehavi et al. Zehavi et al state[95]:

[95] http://www.molecular-cancer.com/content/11/1/44

We show that the expression of miRNAs from a large cluster on human chromosome 14q32 is significantly down-regulated in melanoma cell lines, benign nevi and melanoma samples relative to normal melanocytes. This miRNA cluster resides within a parentally imprinted chromosomal region known to be important in development and differentiation. In some melanoma cell lines, a chromosomal deletion or loss-of-heterozygosity was observed in the cis-acting regulatory region of this cluster.

In several cell lines we were able to re-express two maternally induced genes and several miRNAs from the cluster with a combination of de-methylating agents and histone deacetylase inhibitors, suggesting that epigenetic modifications take part in their silencing.

Stable over-expression of mir-376a and mir-376c, two miRNAs from this cluster that could be re-expressed following epigenetic manipulation, led to modest growth retardation and to a significant decrease in migration in-vitro. Bioinformatic analysis predicted that both miRNAs could potentially target the 3'UTR of IGF1R.

Indeed, stable expression of mir-376a and mir-376c in melanoma cells led to a decrease in IGF1R mRNA and protein, and a luciferase reporter assay indicated that the 3'UTR of IGF1R is a target of both mir-376a and mir-376c. Our work is the first to show that the large miRNA cluster

Note in the above the selection and determination of other miRNAs as well. It is not expected that any single miRNA will be considered the sole controlling element. In fact one may anticipate a progression as the tumor develops. The setting off of miRNAs as the tumor stage changes would be an interesting by-product of this analysis.

Another quite useful analysis of miRNAs and melanoma has been done by Taveira da Cruz and Jasiulionis. In their work the two authors state:

miRNAs are non-coding RNAs that bind to mRNA targets and disturb their stability and/or translation, thus acting in gene posttranscriptional regulation. It is predicted that over 30% of mRNAs are regulated by miRNAs. Therefore these molecules are considered essential in the processing of many biological responses, such as cell proliferation, apoptosis, and stress responsiveness.

As miRNAs participate of virtually all cellular pathways, their deregulation is critical to cancer development. Consequently, loss or gain of miRNAs function may contribute to tumor progression.

Little is known about the regulation of miRNAs and understanding the events that lead to changes in their expression may provide new perspectives for cancer treatment. Among distinct types of cancer, melanoma has special implications. It is characterized as a complex disease, originated from a malignant transformation of melanocytes.

Despite being rare, its metastatic form is usually incurable, which makes melanoma the major death cause of all skin cancers. Some molecular pathways are frequently disrupted in melanoma, and miRNAs probably have a decisive role on these alterations.

Therefore, this review aims to discuss new findings about miRNAs in melanoma fields, underlying epigenetic processes, and also to argue possibilities of using miRNAs in melanoma diagnosis and therapy.

The conclusions drawn from the above paper are considerable. After just a few years there is now a well-accepted understanding of how miRNAs function and that they play critical roles in pathways. However, and this is a very significant however, we do not understand what precipitates them nor do we fully understand their relationship in pathway analysis. What is clear is that they are found in a multiple set of cancers, that that are pathway control elements, but the complex interactions we would anticipate are still unknown.

5.7.7 Measurements of MiRNA

There are many ways in which we can measure miRNAs. The technology has developed greatly in the past decade. An excellent paper by Pritchard et al presents an overview.

Pritchard et al comment on recovery of miRNA. This is an important observation in that it may generally be readily obtained:

It is possible to extract high-quality miRNA from a wide range of cell and tissue sources, including cell lines, fresh tissues, formalin-fixed paraffin-embedded (FFPE) tissues, plasma, serum, urine and other body fluids[27,28] (TABLE 1). The principles for isolating miRNA are, in general, the same as for isolation of total RNA, except that miRNA isolation protocols are often slightly modified to retain (and sometimes to enrich) the small RNA fraction[28]. Widely used commercially available products are generally based on chemical extraction using concentrated chaotropic salts, such as guanidinium thiocyanate (for example, Trizol and QIAzol reagents), followed by a solid-phase extraction procedure on silica columns. miRNAs may also be analysed using total RNA isolated by traditional chemical extraction with Trizol

Pritchard et al summarize the many profiling techniques. Specifically on Figure 3 of their paper (it is worth looking at the paper rather than trying to replicate what they have accomplished) they depict the following:

Approaches to microRNA profiling.

a. Quantitative reverse transcription PCR (qRT-PCR). In TaqMan qRT-PCR, the reverse transcription reactions use stem–loop primers that are specific to the 3! end of the microRNA (miRNA) for specificity (top left). Amplicons are generated using an miRNA-specific forward primer and a reverse primer. As the DNA polymerase proceeds along the template, the TaqMan probe is hydrolysed and fluorescent dye is freed from the quencher, resulting in light emission (top middle). In SYBR-green-based qRT-PCR, miRNA is typically polyadenylated at the 3! end, and oligo-d(T) is used as a reverse transcription primer (bottom left). An miRNA-specific forward primer and a reverse primer that anneals to the 3! portion of the miRNA sequence as

well as to the poly(A) tail enable PCR amplification with dsDNA-intercalating SYBR green dye as the detector (bottom middle). Both TaqMan and SYBR-green-based qRT-PCR are available in 'array' format (right).

b. miRNA microarray. DNA-based capture probes (which may or may not incorporate LNA-modified bases) are used to capture fluorescently tagged miRNAs; this is followed by scanning of slides and quantification of fluorescence.

c. Nanostring nCounter. A bridge oligonucleotide templates ligation of an miRNA to a specific tag. Capture and detection is done by two target-specific probes: a capture probe containing biotin to allow adsorbance to the solid phase via streptavidin and a second reporter probe with an individual colour-coded sequence. No amplification or labelling of miRNA is required with this method.

d. RNA sequencing (RNA-seq). Currently established RNA-seq platforms begin with reverse transcription of miRNA to a cDNA library. Adaptor ligation then allows the library either to be affixed to a solid phase, as in the Illumina platform, or to beads for emulsion PCR, as in the Roche and ABI platforms (for details of sequencing chemistry

5.7.8 MiRNA Melanoma Targets

Profiling of common nevi CN and atypical nevi AN by miRNA has been reported by Holst et al. They conclude:

MicroRNAs (miRNAs) are small non-coding RNAs, which regulate gene expression through base pairing with mRNA and which are crucially involved in carcinogenesis (the so-called oncomiRs). We compared the miRNA signature between acquired melanocytic nevi showing clinical atypia (atypic nevi, AN) and common acquired nevi (common nevi, CN).

We obtained miRNA profiles from 41 biopsies (22 AN and 19 CN) and showed that AN could be differentiated from CN on the basis of the expression of 36 miRNAs (false discovery rate <0.05). OncomiRs were present in this group, and we further confirmed the differential expression of miR-125b and let-7c by qRT-PCR. Our data suggest that miRNAs are functionally involved in the pathogenesis of nevi and possibly malignant melanoma. ...

Our study showed that AN can be differentiated from CN on the basis of the expression of 36 miRNAs. Furthermore, AN showed a tendency to further clustering into two groups, which could define different subtypes of AN. The fact that AN are molecularly heterogeneous has also been suggested by Scantolini et al. (24) on the basis of mRNA expression profiles in melanocytic lesions.

Among the differentially expressed miRNA, we found the known oncomiRs let-7a, let-7c and miR-125b. The expression pattern showing a reduced miR-125b expression indicates a more 'malignant' miRNA signature of AN in comparison with CN. Because N-RAS and P53, known to be involved in MM, can be targeted by these miRNAs (the list of other predicted targets is shown

in the supplementary material), further studies should identify their functional significance in the pathogenesis of melanocytic lesions.

In another paper by Segura et al the authors find:

MiRNA signatures have potential as clinically relevant biomarkers of prognosis in metastatic melanoma. Our data suggest that molecularly based models of risk assessment can improve the standard staging criteria and support the incorporation of miRNAs into such models.

They state:

MiRNAs significantly associated with post-recurrence survival. Eighteen miRNAs found overexpressed in metastatic tissues of patients with longer survival (≥1.5 y) compared with patients with shorter survival (<1.5 y). Fold change, FDR, Cox regression coefficient (d), chromosome location, and host genes (when pertinent) are indicated.

They then present a set of miRNA and calculate their influence.miRNA	Score (d) Cox Regression Coefficient	Location	Host Gene
has-miR-150	−3.98	19q 13.33	Intergenic
has-miR-455-3p	−3.52	9q32	COL27A1
has-miR-145	−3.06	5q 33.1	Intergenic
has-miR-342-3p	−2.9	14q 32.2	EVL
has-miR-497	−2.87	17p 13.1	AC027763.1
has-miR-155	−2.77	21q 21.3	Intergenic
has-miR-342-5p	−2.66	14q 32.2	EVL
has-miR-143	−2.61	5q 33.1	Intergenic
has-miR-193a-3p	−2.43	17q 11.2	Intergenic
has-miR-146b-5p	−2.38	10q 24.32	Intergenic
has-miR-28-3p	−2.32	3q 28	LPP-201
has-miR-10b	−2.14	2q 31.1	HOXD3
has-miR-193b	−2.08	16 p3.12	Intergenic
has-miR-28-5p	−1.87	3q 28	LPP-201
has-miR-142-5p	−1.86	17q 22	Intergenic
has-miR-143	−1.73	5q 33.1	Intergenic
has-miR-126	−1.73	9q34.3	EGFL7
has-miR-214	−1.72	1q 24.3	DNM3

They conclude with:

Many of the miRNAs from our signature are located in genomic regions previously reported as altered in melanoma, such as loss of 9q32 (miR-455-3p) in melanoma cell lines, gain of the 5q locus (miR-145) in acral melanoma, and gain of 21q (miR-155) in uveal melanoma (Supplementary Table S8).

In conclusion, our results show the potential of miRNAs as clinically useful markers of prognosis in metastatic melanoma patients. A six-miRNA signature was able to improve risk stratification for stage III patients, suggesting that miRNAs may serve as a useful molecular adjunct to the current morphologic staging system in identifying high-risk patients who might benefit from adjuvant therapy.

Differential expression of most miRNAs from the predictor signature was also observed in the matched-pair primary tissue, suggesting that the miRNA signature may also play a role in prognosis of early lesions. Further studies with a larger cohort of primary melanoma patients are needed to better define the role of the signature in predicting the development of aggressive disease.

Recent work by Luo also details similar markers (p 66).Luo also details a more complex causative analysis of miR-101. Luo also does a detailed analysis of MITF as well as EZH2 which we have discussed in previous literature analyses. Luo concludes:

To test whether miR-101 is able to down-regulate MITF protein expression, we transfected Ma-Mel-79b and -86b cells with miR-101 mimic and performed Western blot analysis using antibody against MITF. As shown … MITF protein was decreased to 62 % and 56 % in Ma-Mel-79b and -86b cells at 72 hr after transfection of miR-101. We also tested the expression of EZH2, which has been reported to be an oncogene and a target of miR-101 in various types of. Indeed, overexpression of miR-101 down-regulated EZH2 in the two tested melanoma cell lines. Interestingly, overexpression of miR-101 in both cell lines also caused a cleavage of poly-(ADP-ribose) polymerase (PARP) which is one of the targets of caspase-3 and serves as an indicator for apoptosis

Targets for ascertaining prognosis are critical. Not only for melanoma, but they are critical for all types of cancer. miRNAs seems to be such a set of targets.

5.7.9 Therapeutic Application

There is always the desire to find therapeutic methodologies to control cancers and particularly melanomas. With miRNA we have a simple RNA structure, some 22 nucleotides, which can be reproduced or modified to control aberrant pathways. Rather than trying to match to either complex RNAs or proteins we could possibly find more readily targeted miRNAs.

Kota et al present an analysis of the application of miRNA in the area of therapeutics. The have discussed how this may apply to hepatocellular carcinomas. They state:

Therapeutic strategies based on modulation of microRNA (miRNA) activity hold great promise due to the ability of these small RNAs to potently influence cellular behavior. In this study, we investigated the efficacy of a miRNA replacement therapy for liver cancer. We demonstrate that hepatocellular carcinoma (HCC) cells exhibit reduced expression of miR-26a, a miRNA that is normally expressed at high levels in diverse tissues.

Expression of this miRNA in liver cancer cells in vitro induces cell-cycle arrest associated with direct targeting of cyclins D2 and E2. Systemic administration of this miRNA in a mouse model of HCC using adeno-associated virus (AAV) results in inhibition of cancer cell proliferation, induction of tumor-specific apoptosis, and dramatic protection from disease progression without toxicity. These findings suggest that delivery of miRNAs that are highly expressed and therefore tolerated in normal tissues but lost in disease cells may provide a general strategy for miRNA replacement therapies.

There seems to be quite a bit more work being done in this area and it represents an exciting breakthrough segment for therapeutics.

5.7.10 Observations

Much of what we know about miRNAs and their functions has evolved in the past five years to a decade at most. In fact in the past decade one has seen a great opening to RNAs in general. Before that it could be said that RNAs were the poor cousin in the process, the glory given the DNA and then the pathway dynamics dominated by proteins. We now appear to have opened a door on control mechanisms at the RNA level, dominated by miRNA and their control of mRNA before it becomes a protein. Thus RNA is somewhat exciting, and the miRNA have presented an added level of complexity to our modeling of complex cellular dynamics.

Based upon the analysis herein:

The most significant result from the explosion of miRNA effects is that what we have seen as now classic pathways may have significant undercurrent resulting from the miRNAs. Are miRNAs dominant control elements, is so where do they impact the most. We have seen many of the miRNA discoveries as just incidental to studying pathways. In our prior analysis we assumed them to be just noise. Now we can no longer accept such a proposition. In fact they seem to play significant if not dominant roles.

The use of miRNAs as therapeutic targets is of significant interest. We have discussed some of the results and we have tried to place miRNAs in context of a broad therapeutic approach. The true reason is the simplicity of the miRNA structure. It is not a complex protein of hundreds of nucleic acids folded in a complex manner. The miRNA is just some 22 nucleotides on a sugar backbone.

We have been trained to ignore the introns. It was the trash heap of evolution, perhaps of some use in the past. However since miRNAs are intro sourced, we now have a new window on the importance of introns.

We have looked at such proteins as PTEN, p53, and others as the control element. We looked at kinases and receptors and instigating ligands as part of that process. When we examine miRNA we see control coming from within. What instigates the processing and release of miRNAs. What are the feedback loops, if any, between the surface changes on receptors and the activation of miRNAs.

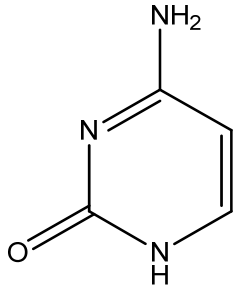
One of the problems we have in many cancers is both diagnosis and prognosis. In melanoma unfortunately prognosis may often be dire, but not always. In addition diagnosis of pigmented lesions is often problematic. Take a simple melanoma in situ, where it is diagnosed based on upward movement of the melanocyte. Are there differences in the MIS? Namely is each MIS identical, just losing its stability, say through loss of E-cadherin, and if not are there simple miRNAs which can be targeted and profiled.

There are many more observations which will evolve as we better understand miRNAs. Since we are at the beginning of understanding them we must keep in mind the ever changing field of play, and thus any analysis must include miRNAs as significant participants.

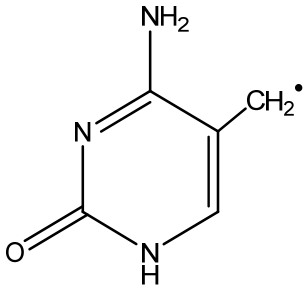
5.8 METHYLATION

DNA methylation is a process whereby the cytosine is changed by the insertion of a methyl group on the 5 carbon of the ring. It is a process which is epigenetic and can dramatically modify gene expression. In fact many of the methylation issue in humans are also common to plants, see the work by Zuberman. There has been a great deal of work demonstrating the impact of methylation on cancer progression. Specifically the recent summary by Herman and Baylin, that of Palii and Robertson, that od Robertson and Wolffe, Strathdee and Brown, Calin and Croce.

Basic cytosine is shown below. It has two NH groups at opposite poles and a single oxygen.



Now when the 5 carbon is replaced by a methyl group we obtain the form below. This is methylated cytosine.



+

As is stated in the paper by Miranda and Jones:

DNA methylation is a covalent modification in which the 5o position of cytosine is methylated in a reaction catalyzed by DNA methyltransferases (DNMTs) with S-adenosyl-methionine as the methyl donor.

In mammals, this modification occurs at CpG dinucleotides and can be catalyzed by three different enzymes, DNMT1, DMNT3a, and DNMT3b.DNAmethylation plays a role in the long-term silencing of transcription and in heterochromatin formation.

As an epigenetic modification, DNA methylation permits these silenced states to be inherited throughout cellular divisions.

We continue with the discussion in Mirand and Jones as follows:

Silencing of genetic elements can be successfully initiated and retained by histone modifications and chromatin structure. However, these modifications are easily reversible making them make poor gatekeepers for long-term silencing. Therefore, mammalian cells must possess an additional mechanism for prolong silencing of these sequences. An important component of this process is DNA methylation. DNA methylation is a stable modification that is inherited throughout cellular divisions.

When found within promoters, DNA methylation prevents the reactivation of silent genes, even when the repressive histone marks are reversed. This allows the daughter cells to retain the same expression pattern as the precursor cells and is important for many cellular processes including the silencing of repetitive elements, X-inactivation, imprinting, and development.

We now present a key Figure from Miranda and Joner regarding the methylated reading of DNA. They state regarding the Figure below:

Chromatin structure of CpG islands and CpG poor regions in healthy cells and during cancer. In healthy cells, CpG islands are generally hypomethylated. This allows for an open chromatin structure. However, the CpG poor regions found in repetitive elements within the intergenic and intronic regions of the genome are methylated and thereby maintain a closed chromatin structure. In cancer and on the inactive X chromosome many CpG islands become methylated, forcing these regions into a closed chromatin structure.

When CpG islands located within promoters are methylated, the corresponding genes are persistently silenced. In contrast, the CpG poor regions become hypomethylated allowing for an open chromatin structure.

As Robertson states:

It is now clear that the genome contains information in two forms, genetic and epigenetic. The genetic information provides the blueprint for the manufacture of all the proteins necessary to create a living thing while the epigenetic information provides instructions on how, where, and when the genetic information should be used.

Ensuring that genes are turned on at the proper time is as important as ensuring that they are turned off when not needed.

The major form of epigenetic information in mammalian cells is DNA methylation, or the covalent addition of a methyl group to the 5-position of cytosine predominantly within the CpG dinucleotide. DNA methylation has profound effects on the mammalian genome.

Some of these effects include transcriptional repression, chromatin structure modulation, X chromosome inactivation, genomic imprinting, and the suppression of the detrimental effects of repetitive and parasitic DNA sequences on genome integrity.

Robertson then proceeds to detail the genes impacted by hypermethylation. We summarize them below:

Gene	*Function*
pRb	Regulator of G1/S phase transition
p16 INK4a	Cyclin-dependent kinase inhibitor
p15 INK4b	Cyclin-dependent kinase inhibitor
ARF	Regulator of p53 levels
hMLH1	DNA mismatch repair
APC	Binds b-catenin, Regulation of actin cyto-skeleton?
VHL	Stimulates angiogenesis
BRCA1	DNA repair
LKB1	Serine/threonine protein kinase
E-cadherin	Cell ± cell adhesion
ER	Transcriptional activation of estrogen-responsive genes
GSTP1	Protects DNA from oxygen radical damage
O6-MGMT	Repair/removal of bulky adducts from guanine
TIMP3	Matrix metallo proteinase inhibitor
DAPK1	Kinase required for induction of apoptosis by g interferon
p73	Apoptosis?, structurally similar to p53

Regarding PIN, the one which is most concern is the GSTP1 gene and its suppression allowing for DNA damage from inflammation and oxygenation damage.

In the context of cancer generation and progression, the epigenetic effect of hyper and hypo methylation are best described by Esteller:

The low level of DNA methylation in tumors as compared with the level of DNA methylation in their normal-tissue counterparts was one of the first epigenetic alterations to be found in human cancer.

The loss of methylation is mainly due to hypomethylation of repetitive DNA sequences and demethylation of coding regions and introns −regions of DNA that allow alternative versions of the messenger RNA (mRNA) that is transcribed from a gene. A recent large-scale study of DNA methylation with the use of genomic microarrays has detected extensive hypo-methylated genomic regions in gene-poor areas.

During the development of a neoplasm, the degree of hypomethylation of genomic DNA increases as the lesion progresses from a benign proliferation of cells to an invasive cancer.

Three mechanisms have been proposed to ex-plain the contribution of DNA hypomethylation to the development of a cancer cell:

(i) generation of chromosomal instability,

(ii) reactivation of transposable elements, and

(iii) loss of imprinting.

Under methylation of DNA can favor mitotic recombination, leading to deletions and translocations, and it can also promote chromosomal rearrangements. This mechanism was seen in experiments in which the depletion of DNA methylation by the disruption of DNMTs caused aneuploidy. Hypomethylation of DNA in malignant cells can reactivate intra-genomic endo-parasitic DNA.

5.8.1 Overview

DNA methylation is a process whereby the cytosine is changed by the insertion of a methyl group on the 5 carbon of the ring. It is a process which is epigenetic and can dramatically modify gene expression. In fact many of the methylation issue in humans are also common to plants, see the work by Zilberman. There has been a great deal of work demonstrating the impact of methylation on cancer progression; specifically the recent summary by Herman and Baylin, that of Palii and Robertson, that of Robertson and Wolffe, Strathdee and Brown, Calin and Croce, are all worth reviewing.

In this report we examine methylation and its impact on several cancers. We will also examine briefly the causes of methylation as well as the therapeutics in use to modulate cancers that cause or persistence is supported by methylation related products, either directly or indirectly.

In the paper by Das and Singal, the authors define epigenetics in a quite clear manner:

Epigenetics can be described as a stable alteration in gene expression potential that takes place during development and cell proliferation, without any change in gene sequence.

DNA methylation is one of the most commonly occurring epigenetic events taking place in the mammalian genome. This change, though heritable, is reversible, making it a therapeutic target.

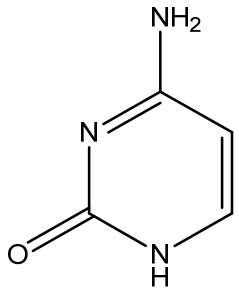
Epigenetics has evolved as a rapidly developing area of research.

Recent studies have shown that epigenetics plays an important role in cancer biology, viral infections, activity of mobile elements, somatic gene therapy, cloning, transgenic technologies, genomic imprinting, developmental abnormalities, mental health, and X-inactivation

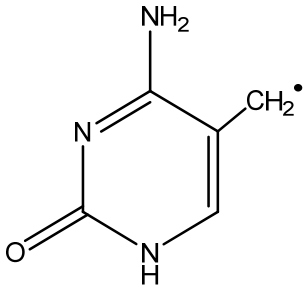
This is one of the clearest definitions of epigenetics and especially the linking of methylation to epigenetics. The classic Watson and Crick model, now some 60 years old, we had the paradigm of DNA, RNA and protein. It was the proteins which did the work. In the 1953 world the proteins stood one by one and the clarity of gene to protein was unquestioned. Yet as we have

come to better understand the details, and the details always count, there are many interfering epigenetic factors that all too often get in the way. Methylation is but one of those factors.

Basic cytosine is shown below. It has two NH groups at opposite poles and single oxygen.



Now when the 5 carbon is replaced by a methyl group we obtain the form below. This is methylated cytosine.



Thus this small change in C, by adding the methyl group, can make for a dramatic difference in the expression of genes. For example a well-controlled gene for proliferation, such as PTEN, may have its control over-ridden by the methylation of Introns of CpG islands, namely collections of C, cytosine nucleotides, and G, guanine nucleotides. The introns may be down from the gene, they may even be on a promoter section. The impact could aberrant cell proliferation and growth.

We examine the process; we then look at three types of cancers, a glandular, an epidermal, and a hematopoietic form and then examine some means used to control those cancers through the understanding or methylation and the control of it by therapeutics designed just for that purpose.

What is important about understanding methylation and especially all epigenetic changes is that it may perhaps be simpler to control them rather than a gene mutation. As Brower states:

The move from a purely genetic to an epigenetic model is crucial for prevention strategies. As numerous gene therapy trials have shown, it is very difficult to treat a genetic disease by re-activating the dormant, mutated gene or by replacing it with a non-mutated one. "Epigenetic changes are reversible, and therefore have an edge over genetics," says Mukesh Verma, an epigeneticist at the National Cancer Institute's division of cancer control and population

sciences in Bethesda, Maryland. Furthermore, epigenetic changes in cancer occur before genetic mutations. "If you can prevent methylation of those tumour suppressor genes, you might have a valuable prevention strategy," says Baylin.

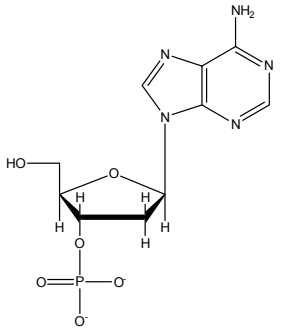
Thus if we see cancers when they are driven by methylation, then can we actually anticipate reversing the process by reversing the methylation changes. Thus with prostate cancer can we anticipate a preventative measure as one increasing certain methylation preventative therapeutics, can we do the same with say MDS, and can we attempt to do the same with say a melanoma. This is what we examine herein.

5.8.2 Some DNA Basics

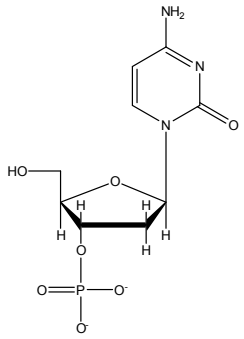
We begin with some simple facts about DNA and then we lead to the methylation of cytosine. But first, the basics of DNA.

DNA is composed of just five basis elements; a ribose backbone with phosphates, and four different nucleotides (C, G, A and T). They align in a double stranded classic DNA pattern.

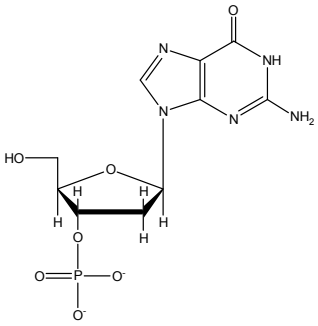
The base pairs and their ribose/phosphate backbone parts are shown below.



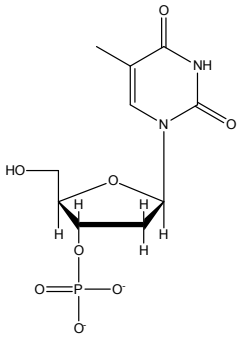
Adenine



Cytosine

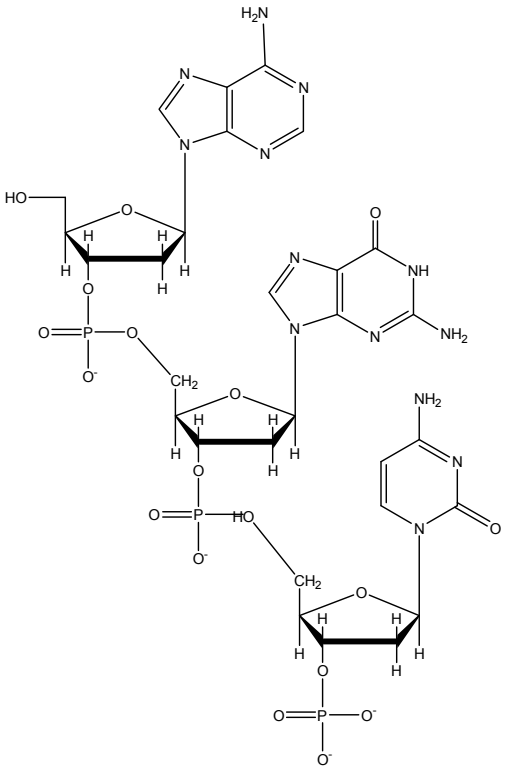


Guanine



Thymine

Now we connect these in the one side of the double helix as is shown below:

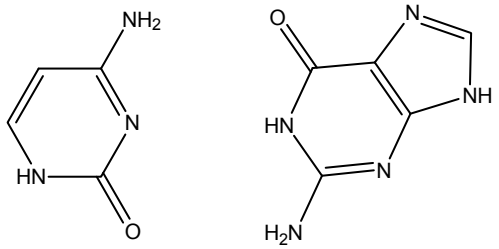


Then from here we can connect the A-T and G-C pairs which make up the DNA as we know it.

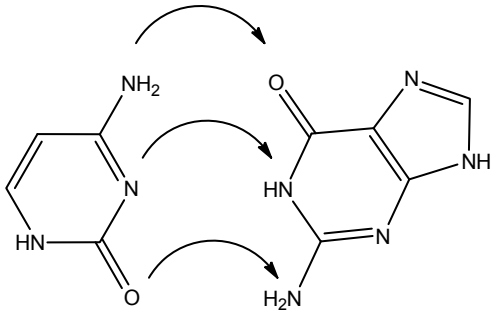
The key observation of Watson and Crick was the hydrogen bonding between base pairs. As Watson and Crick stated in 1953:

The novel feature of the structure is the manner in which the two chains are held together by the purine and pyrimidine bases. The planes of the bases are perpendicular to the fibre axis. They are joined together in pairs, a. single base from ***one chain being hydrogen-bonded to a single base from the other chain****, so that the two lie side by side with identical z-co-ordinates. One of the pair must be a purine and the other a pyrimidine for bonding to occur. The hydrogen bonds are made as follows: purine position 1 to pyrimidine position 1; purine position 6 to pyrimidine position 6.*

The CG bonding is shown below:



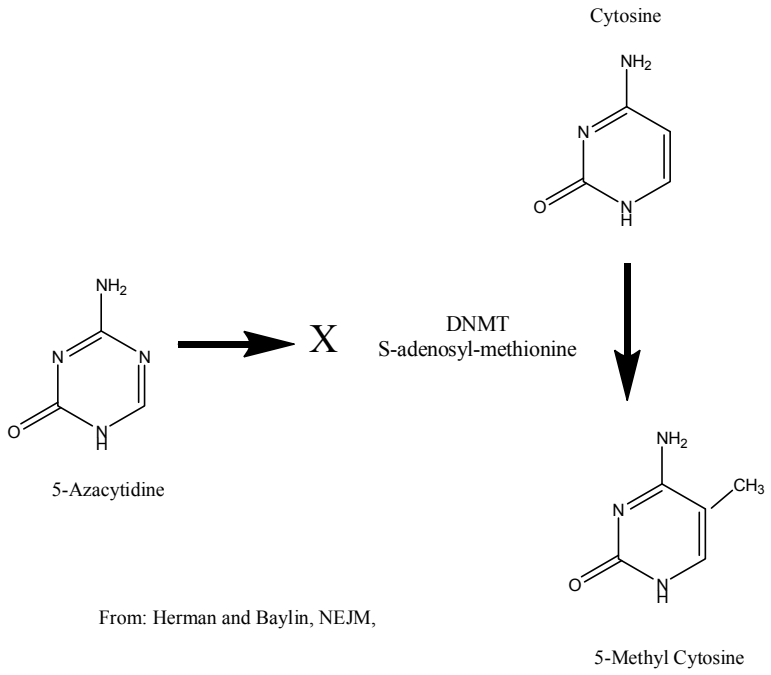
Hydrogen Bonding between C and G



This model is the basis of what we now know as our DNA. The DNA is divided into chromosomes, different strands, and then in the chromosomes we have Introns, non-expressing parts of DNA, and Exons, the expressing parts. The current view is that although the non-expressing parts do not yield proteins they strongly affect that process. That is what methylation does.

5.8.3 Methylation at the Molecule

What is methylation? Simply, the attachment of a methyl group to the cytosine molecule creates a methylated C. This is not a complicated process but one which happens frequently and may have significant effects. Cytosine gets methylated and is converted to 5-methyl cytosine. This is accomplished by means of two enzymes as depicted below. This occurs when we have a C and G adjacent. It occurs to the C in that pair. We depict that transition below. Note also that by using 5-Azacytadine we can block that transition.



From: Herman and Baylin, NEJM,

Now there are the CpG islands. These are C, cytosine, and G, guanine, adjacent nucleotides which are connected via a phosphodiester bone between the two, and multiple collections of these paired nucleotides. The CpG island is then an area dense in these CG pairs connected by the phosphodiester bond, but the "island" may contain nucleotides other than the CG pairs, but generally are high in CG pair concentration, usually more than 50%.

One should note that the statistical probability of such large CG pairings would normally be quite low. One would anticipate equal probability for any nucleotide and any nucleotide pairing. Furthermore such a high concentration is statistically extremely rare but if often existentially quite common.

The CpG islands may be from 300 to over 3,000 base pairs in total length, and are frequently found in gene promoter regions. Thus when the CpG islands are methylated, namely the C is methylated, then the island gets silenced as does the corresponding gene. Namely methylation of CpG islands can result in gene silencing. This then becomes a critical issue if the gene is a control gene such as PTEN, p53, or many of the critical pathway control genes. The CpG islands are also propagated to cell progeny during mitosis, thus a methylated island remains so in the cells progeny.

However understanding methylation of islands, and having a means to demethylate the islands may present a reasonable way to develop therapeutics for cancers resulting from methylated regions. We shall examine that shortly.

As Laird and Jaenisch state:

The normal pattern of 5-methylcytosine distribution DNA methylation in mammals is found as a covalent modification at the fifth carbon position of cytosine residues within CpG dinucleotides. Most of the CpG dinucleotides in the human genome are methylated.

However, 5-methylcytosine makes up less than 1% of all nucleotides, since CpG dinucleotides are under-represented about five-fold in the mammalian genome. The paucity of CpG dinucleotides in the mammalian genome is attributed to a higher mutation rate of methylated versus unmethylated cytosine residues.

CpG dinucleotides and 5-methylcytosine are unevenly distributed in the genome. Most of the genome is heavily methylated with a corresponding deficit in CpG dinucleotides. About 1 to 2% of the genome consists of islands of non-methylated DNA and these sequences show the expected frequency of CpG dinucleotides.

CpG islands are about 1 kb long and are not only CpG-rich, but generally G/C-rich as well and are found at the 5' end of genes. All known housekeeping genes and some tissue-specific genes have associated CpG islands.

5.8.4 Methylation and Gene Expression

We now want to discuss methylation and gene expression. Reference will be made to the work of Herman and Baylin, Jones and Takai, McCabe et al, Allis et al, and Issa and Kantarjian.

We begin with Herman and Baylin and their description of the diagram below:

In most of the mammalian genome, which is depicted here as exons 1, 2, and 3 of a sample gene (boxes 1, 2, and 3), introns of the gene (line between the exons), and regions outside the gene, the CpG dinucleotide has been depleted during evolution, as shown by the small number of such sites (circles).

Small regions of DNA, approximately 0.5 to 4.0 kb in size, harbor the expected number of CpG sites and are termed CpG islands. Most of these are associated with promoter regions of approximately half the genes in the genome (numerous circles surrounding and within exon 1 of the sample gene). In normal cells, most CpG sites outside of CpG islands are methylated (black circles), whereas most CpG-island sites in gene promoters are unmethylated (white circles).

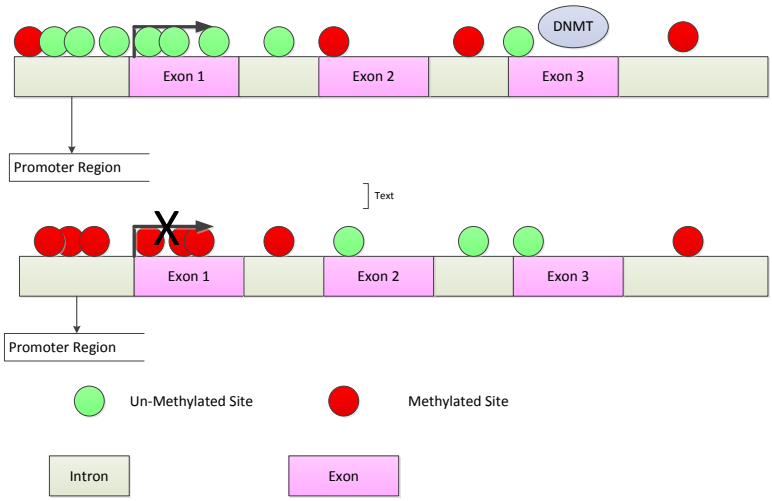
This methylated state in the bulk of the genome may help suppress unwanted transcription, whereas the unmethylated state of the CpG islands in gene promoters permits active gene

transcription (arrow in upper panel). In cancer cells, the DNA-methylation and chromatin patterns are shifted.

Many CpG sites in the bulk of the genome and in coding regions of genes, which should be methylated, become unmethylated, and a growing list of genes have been identified as having abnormal methylation of promoters containing CpG islands, with associated transcriptional silencing (red X at the transcription start site).

Although there are possible explanations and findings from ongoing investigations, it is not known why the DNA-methylating enzymes fail to methylate where they normally would and which of these enzymes are mediating the abnormal methylation of CpG islands in promoters.

We depict a modified version of their Figure below:



Thus methylation in this case blocks the expression of the targeted gene.

5.8.5 Methylation and Deamination (C to T)

Methylation may also progress to more dramatic changes. We discuss here the change of C to T, a serious change in a DNA base pair which can result in dramatic changes in gene expression.

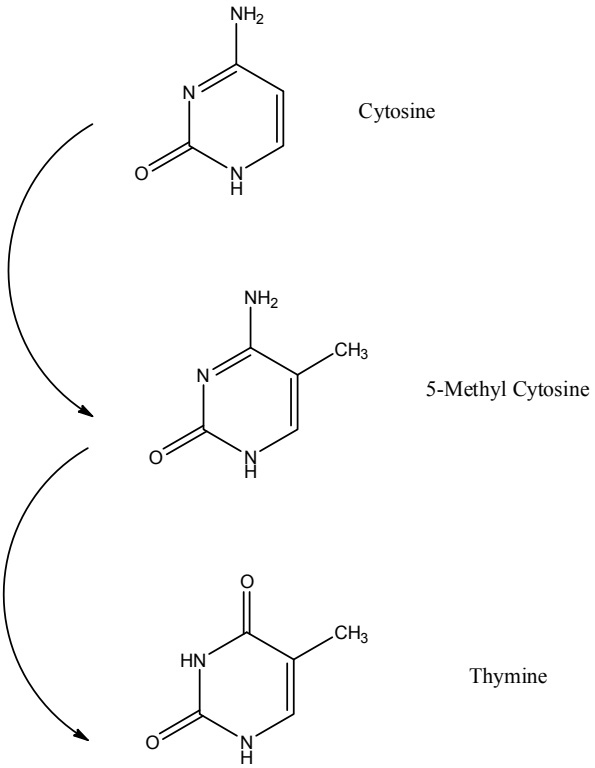
As Herman and Baylin state:

Although only four bases — adenine, guanine, cytosine, and thymine — spell out the primary sequence of DNA, there is a covalent modification of postreplicative DNA (i.e., DNA that has replicated itself in a dividing cell) that produces a "fifth base." Reactions using S -adenosyl-methionine as a methyl donor and catalyzed by enzymes called DNA methyltransferases (DNMTs) add a methyl group to the cytosine ring to form methyl cytosine.

In humans and other mammals, this modification is imposed only on cytosines that precede a guanosine in the DNA sequence (the CpG dinucleotide). The overall frequency of CpGs in the genome is substantially less than what would be mathematically predicted , probably because DNA methylation has progressively depleted the genome of CpG dinucleotides over the course of time.

The mechanism of the depletion is related to the propensity of methylated cytosine to deaminate, thereby forming thymidine. If this mutation is not repaired, a cytosine-to-thymidine change remains.

The depletion of CpG dinucleotides in the genome corresponds directly to sites of such nucleotide transitions, and this change is the most common type of genetic polymorphism (variation) in human populations.



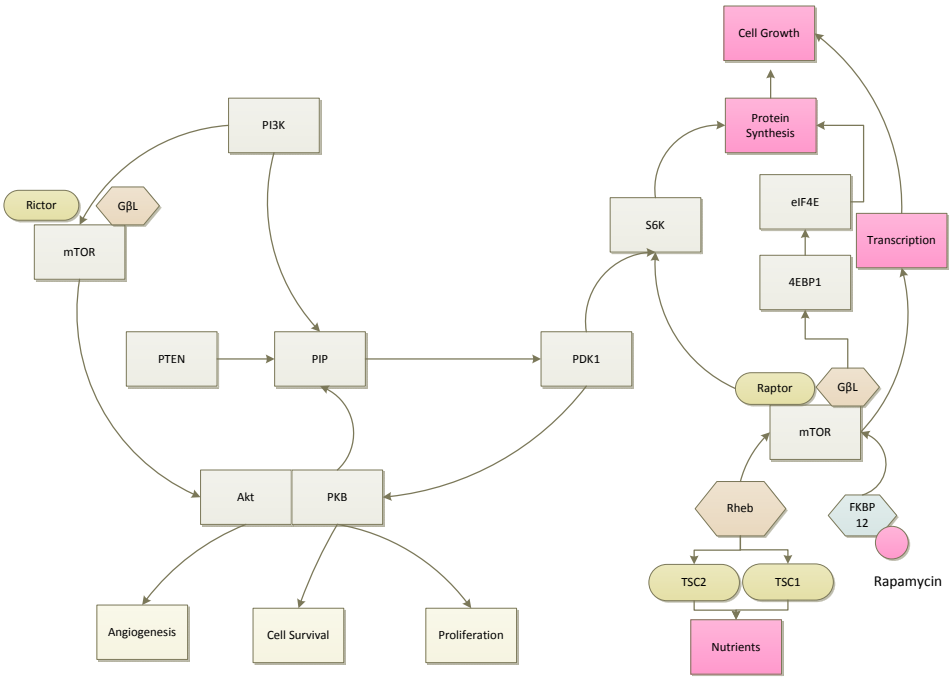
From Robertson (2001) we have some of the genes influenced by methylation or as he states:

CpG-island-associated genes involved in cell growth control or metastasis that can become hypermethylated and silenced in tumors.

We depict the Table below from Robertson on some of the genes impacted by this type of methylation. Most of these are significant regulatory genes.

Gene	*Function*
pRb	Regulator of G1/S phase transition
$p16^{INK4a}$	Cyclin-dependent kinase inhibitor
$p15^{INK4b}$	Cyclin-dependent kinase inhibitor
ARF	Regulator of p53 levels
hMLH1	DNA mismatch repair
APC	Binds β-catenin, Regulation of actin cytoskeleton?
VHL	Stimulates angiogenesis
BRCA1	DNA repair
LKB1	Serine/threonine protein kinase
E-cadherin	Cell-cell adhesion
ER	Transcriptional activation of estrogen-responsive genes
GSTPI	Protects DNA from oxygen radical damage
0^6-MGMT	Repair/removal of bulky adducts from guanine
TIMP3	Matrix metalloproteinase inhibitor
DAPK1	Kinase required for induction of apoptosis by γ interferon
p73	Apoptosis structurally similar to p53

For example we show below some typical pathways and the above genes are seen targeted by methylation.



Methylation may then interfere with many of the genes in the above pathways.

5.8.6 Causes of Methylation

The major question which is often asked is what causes methylation. In Allis et al on p 460 the authors discuss some of the putative cause of methylation and methylation related cancers. Although not confirmative it is consistent with clinical correlations as well.

As Issa and Kartarjian state:

Much remains to be learned about the causes of DNA methylation abnormalities in cancer; for the most part, methylation seems to be gene specific. In some cases, a rare methylation event appears in cancer because of selection , while in others methylation anomalies are downstream of an oncogenic event …

As McCabe et al state:

DNA methylation patterns in human cancer cells are considerably distorted. Typically, cancer cells exhibit hypomethylation of intergenic regions that normally comprise the majority of a cell's methyl-cytosine content . Consequently, transposable elements may become active and contribute to the genomic instability observed in cancer cells.

Simultaneously, cancer cells exhibit hypermethylation within the promoter regions of many CpG island-associated tumor suppressor genes, such as the retinoblastoma gene (RB1), glutatione S-transferase pi (GSTP1), and E-cadherin (CDH1). As a result, these regulatory genes are transcriptionally silenced resulting in a loss-of-function. Thus, through the effects of both hypo- and hyper-methylation, DNA methylation significantly affects the genomic landscape of cancer cells, potentially to an even greater extent than coding region mutations, which are relatively rare

McCabe et al continue:

Although the precise molecular mechanisms underlying the establishment of aberrant DNA hypermethylation remain elusive, recent studies have identified some contributing etiologic factors.

For example, chronic exposure of human bronchial epithelial cells to ***tobacco-derived carcinogens drives hypermethylation*** *of several tumor suppressor genes including CDH1 and RASSF2A.*

Stable knockdown of DNMT1 prior to carcinogen exposure prevented methylation of several of these genes indicating a necessary role for this enzyme in the molecular mechanism underlying hypermethylation.

The reactive oxygen species (ROS) associated with chronic inflammation is another source of DNA damage with the potential to affect DNA methylation as halogenated pyrimidines, one form

of ROS-induced damage, mimic 5-methylcytosine and stimulate DNMT1-mediated CpG methylation in vitro and in vivo.

Indeed, study of the glutatione peroxidase 1 and 2 double knockout model of inflammatory bowel disease found that 60% of genes that are hypermethylated in colon cancers also exhibit aberrant methylation in the inflamed noncancerous precursor tissues. Although the mechanisms by which DNA damage mediates DNA methylation are not fully understood, O'Hagan and colleagues have examined the process with an engineered cell culture model in which a unique restriction site was incorporated into the CpG island of the E-cadherin promoter.

Thus the actual molecular mechanics leading to methylation are not fully understood but like most cancers inflammation appears to be a driving factor. What the cause of that inflammation may be is not yet clear.

5.8.7 Methylation Effects on DNA

As is stated in the paper by Miranda and Jones:

DNA methylation is a covalent modification in which the 5₀position of cytosine is methylated in a reaction catalyzed by DNA methyltransferases (DNMTs) with S-adenosyl-methionine as the methyl donor.

In mammals, this modification occurs at CpG dinucleotides and can be catalyzed by three different enzymes, DNMT1, DMNT3a, and DNMT3b.DNAmethylation plays a role in the long-term silencing of transcription and in heterochromatin formation.

As an epigenetic modification, DNA methylation permits these silenced states to be inherited throughout cellular divisions.

We continue with the discussion in Mirand and Jones as follows:

Silencing of genetic elements can be successfully initiated and retained by histone modifications and chromatin structure. However, these modifications are easily reversible making them make poor gatekeepers for long-term silencing. Therefore, mammalian cells must possess an additional mechanism for prolong silencing of these sequences. An important component of this process is DNA methylation. DNA methylation is a stable modification that is inherited throughout cellular divisions.

When found within promoters, DNA methylation prevents the reactivation of silent genes, even when the repressive histone marks are reversed. This allows the daughter cells to retain the same expression pattern as the precursor cells and is important for many cellular processes including the silencing of repetitive elements, X-inactivation, imprinting, and development.

We now present a key Figure from Miranda and Joner regarding the methylated reading of DNA. They state regarding the Figure below:

Chromatin structure of CpG islands and CpG poor regions in healthy cells and during cancer. In healthy cells, CpG islands are generally hypomethylated. This allows for an open chromatin structure. However, the CpG poor regions found in repetitive elements within the intergenic and intronic regions of the genome are methylated and thereby maintain a closed chromatin structure. In cancer and on the inactive X chromosome many CpG islands become methylated, forcing these regions into a closed chromatin structure.

When CpG islands located within promoters are methylated, the corresponding genes are persistently silenced. In contrast, the CpG poor regions become hypomethylated allowing for an open chromatin structure.

As Robertson states:

It is now clear that the genome contains information in two forms, genetic and epigenetic. The genetic information provides the blueprint for the manufacture of all the proteins necessary to create a living thing while the epigenetic information provides instructions on how, where, and when the genetic information should be used.

Ensuring that genes are turned on at the proper time is as important as ensuring that they are turned off when not needed.

The major form of epigenetic information in mammalian cells is DNA methylation, or the covalent addition of a methyl group to the 5-position of cytosine predominantly within the CpG dinucleotide. DNA methylation has profound effects on the mammalian genome.

Some of these effects include transcriptional repression, chromatin structure modulation, X chromosome inactivation, genomic imprinting, and the suppression of the detrimental effects of repetitive and parasitic DNA sequences on genome integrity.

Robertson then proceeds to detail the genes impacted by hypermethylation. We summarize them below:

Gene	*Function*
pRb	Regulator of G1/S phase transition
p16 INK4a	Cyclin-dependent kinase inhibitor
p15 INK4b	Cyclin-dependent kinase inhibitor
ARF	Regulator of p53 levels
hMLH1	DNA mismatch repair
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VHL	Stimulates angiogenesis
BRCA1	DNA repair
LKB1	Serine/threonine protein kinase
E-cadherin	Cell ± cell adhesion
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O6-MGMT	Repair/removal of bulky adducts from guanine
TIMP3	Matrix metallo proteinase inhibitor
DAPK1	Kinase required for induction of apoptosis by g interferon
p73	Apoptosis?, structurally similar to p53

Regarding PIN, the one which is most concern is the GSTP1 gene and its suppression allowing for DNA damage from inflammation and oxygenation damage.

In the context of cancer generation and progression, the epigenetic effect of hyper and hypo methylation is best described by Esteller:

The low level of DNA methylation in tumors as compared with the level of DNA methylation in their normal-tissue counterparts was one of the first epigenetic alterations to be found in human cancer.

The loss of methylation is mainly due to hypomethylation of repetitive DNA sequences and demethylation of coding regions and introns −regions of DNA that allow alternative versions of

the messenger RNA (mRNA) that are transcribed from a gene. A recent large-scale study of DNA methylation with the use of genomic microarrays has detected extensive hypo-methylated genomic regions in gene-poor areas.

During the development of a neoplasm, the degree of hypomethylation of genomic DNA increases as the lesion progresses from a benign proliferation of cells to an invasive cancer.

Three mechanisms have been proposed to ex-plain the contribution of DNA hypomethylation to the development of a cancer cell:

(i) generation of chromosomal instability,

(ii) reactivation of transposable elements, and

(iii) loss of imprinting.

Under methylation of DNA can favor mitotic recombination, leading to deletions and translocations, and it can also promote chromosomal rearrangements. This mechanism was seen in experiments in which the depletion of DNA methylation by the disruption of DNMTs caused aneuploidy. Hypomethylation of DNA in malignant cells can reactivate intra-genomic endo-parasitic DNA.

1.1.1.4 Hypomethylation

As Laird and Jaenisch state:

Hypomethylation: Reduced levels of global DNA methylation have been reported for a variety of malignancies in the past decade. Gama Sosa and coworkers found that in a wide variety of tumors, hypomethylation not only correlated with transformation, but also with tumor progression . In their analysis, only 7% of 43 normal tissues had a 5-methylcytosine content below 0.8 mol%, whereas 10% of 21 benign tumors, 27% of 62 primary malignancies and 60% of 20 secondary malignancies had a 5-methylcytosine content below 0.8 mol%. On the other hand, Feinberg and coworkers did not find a further reduction in DNA methylation levels in the progression from benign to malignant colonic neoplasia, suggesting an early role for DNA hypomethylation in colorectal cancer

1.1.1.5 Hypermethylation

As again with Laird and Jaenisch we have:

Hypermethylation: There have also been many reports of regional increases in DNA methylation levels. Baylin and coworkers have found regional hotspots for hypermethylation on chromosomes 3p, 11p and 17p in a variety of human tumors. These include CpG island areas that are normally never methylated in vivo, but are found to be methylated in tumor tissues. This is reminiscent of the changes that occur at CpG islands at non-essential genes in tissue culture. Baylin's group has dissected the sequential order of hypermethylation events in an in vitro model for lung tumor progression. There is evidence for inactivation of tumor-suppressor gene function

through hypermethylation of the Rb gene in sporadic retinoblastoma. Transient transfection experiments showed that specific hypermethylation in the promoter region of Rb could reduce expression to 8% of an unmethylated control. It is possible, therefore, that hypermethylation of tumor-suppressor genes leading to gene inactivation results in a selective growth advantage of the transformed cells.

5.8.8 Methylation and Cancer

We now examine the impact of methylation on several cancers. We have selected three different types:

1. Glandular: This is prostate cancer. Many adenocarcinomas are typical of this type. Glands seem often to be the source of cancers and one could surmise it is because they are continually active cell sites with high mitotic activity.

2. Epidermal: We select melanoma as an example. This is especially interesting because it is a cancer which is often attributed to UV radiation, since the melanocytes are so close to the skin surface, a few dozen keratinocytes deep.

3. Hematopoietic: The majority of blood/bone generated cancers result from a variety of changes. CML is a classic model with a Philadelphia chromosome abnormality, a translocation. MDS, myelodysplastic syndrome, on the other hand, is a pre-cancerous state where hypermethylation is the driving factor. This is interesting in that unlike CML which has a clear genetic change, MDS has a clear hypermethylated state. It may result in a genetic change and thus AML but the progression may be mitigated by drugs which mitigate methylation.

We examine the literature on each as regards to methylation impact.

1.1.1.6 Prostate

Prostate cancer is a complex malignancy of a glandular element. It may be indolent or highly aggressive, and at this time it is quite difficult to determine the difference based solely on pathology examination. One of the themes we shall see in methylation and cancers will be exogenous effects such as sunlight in melanoma, such as chemicals and radiation in MDS and such as free radicals and infections in prostate cancer. These factors all seem to impact methylation.

In a recent (2013) paper by Vasiljevic et al they state:

Our data indicate CpG methylation of the first HSPB1 intron to be an important biomarker that identifies aggressive PCas otherwise regarded as low risk by current clinical criteria but that, biologically, require immediate active management.

This is a very powerful conclusion. It is a step to identifying indolent from aggressive. They continue:

Heat shock protein 27 (Hsp-27), encoded by the gene HSPB1 located on chromosome 7q11.23 has been shown in several independent studies to be a reliable biomarker of poor clinical outcome in human prostate cancer (PCa) as well as in human breast cancer, colorectal cancer and malignant melanoma.

Biologically, Hsp-27 is an anti-apoptotic protein that induces intracellular homeostasis and allows cellular repair and recovery after physical and chemical insults. Although Hsp-27 is constitutively expressed in most human cells, induced overexpression during carcinogenesis can lead to increased survival of the malignant cells.

Therefore, it is not surprising that studies link high expression of Hsp-27 to unfavorable prognosis in many cancer types. The prognostic potential has been confirmed in prostate cell lines14 as well as in prostate tissues where overexpression has been linked with hormone resistance and poor clinical outcome.

During early prostate carcinogenesis, expression of Hsp-27 protein becomes universally abrogated but may be re-expressed subsequently, in which case the malignancy develops an aggressive phenotype.

Although the specific factors controlling these changes are presently unknown, one plausible mechanism is DNA methylation (DNAme) of the HSPB1 gene. The majority of CpG dyads in the human genome are methylated with the exception of CG-rich regions called CpG islands.16 CpG islands mainly cover gene promoters and first exons and their hypermethylation is associated with repressed transcription of many tumor-suppressor genes.

Therefore, we test the hypothesis that the DNAme status of HSPB1, particularly the HSPB1 promoter, exon and intron regions, is an important determinant of PCa behavior.

Thereafter, we assess any potential relationship between DNAme and Hsp-27 protein levels. Our objectives are also to investigate the diagnostic biomarker potential, by comparing the methylation status of BPH vs PCa, and the prognostic potential of DNAme, by analyzing the association between the methylation and PCa-specific death in the well-characterized Transatlantic Prostate Group (TAPG) cohort.

They conclude:

In conclusion, HSPB1 is essentially unmethylated in BPH but with increasing neoplastic changes through to PCa, the gene becomes increasingly methylated, proceeding from the promoter in a 3' direction. In PCas with low Gleason score, higher methylation within the HSPB1 gene independently identifies patients with poor clinical outcome and hence is an objective biomarker identifying the immediate need for active intervention in the clinical management of this cohort of patients.

This is a powerful observation and sets the path for improved prognostics on PCa.

In an older paper by O'Shaughnessey et al they state:

PIN and prostate cancer lesions share a number of somatic genome abnormalities, including loss of DNA sequences at 8p and increased GSTP1 CpG island DNA methylation, among others. Finally, transgenic mouse strains prone to developing prostate cancers typically develop PIN lesions in advance of the appearance of invasive cancer.

We have discussed elsewhere the HGPIN issue regarding PCa and the questions raised by the assumed linear progression from HGPIN top PCa, except in certain cases where we hypothesize the removal of stem calls upon biopsy.

1.1.1.7 Melanoma

Melanoma is a solid tumors which has the tendency to metastasize very rapidly. Melanoma is fundamentally a malignancy of the melanocytes and the melanocytes are often changed into a malignant state due to their proximity to the skin surface, at the basal layer of the epidermis, and the impact of UV light on their progress. We have argued elsewhere that methylation of portions of the DNA due to such factors as backscatter radiation may be a significant factor as well. The skin being so thin absorbs the radiation more strongly that the viscera and thus is millions of times more sensitive.

From Bennett we have:

A primary event in progression would be a cellular change that is clonally inherited, that contributes to the eventual malignancy, and that occurs independently rather than as a secondary result of some other oncogenic change.

*These events are either genetic (gene mutation, deletion, amplification or translocation), or epigenetic (a heritable change other than in the DNA sequence, **generally transcriptional modulation by DNA methylation** and/or by chromatin alterations such as histone modification). In clonal evolution of cancer, such a primary event would initiate a new, more progressed, clone with a growth advantage over its neighbors, or an alternative selective advantage such as migration…. The β-catenin pathway can be upregulated by several kinds of primary and secondary changes in melanoma. These include uncommon activating mutations of b-catenin (CTNNB1) itself, **methylation or mutation of APC**, overexpression of proto-oncoprotein SKI….*

In a recent paper by Mazar et al they report on melanoma as follows:

Here, we report that cell lines derived from malignant melanomas and melanoma patient samples have hypermethylated CpG islands in the 59-upstream regions of several miRNA coding genes, including that of miR-34b. We engineered two cell lines derived from metastatic melanoma to ectopically express miR-34b, and show that these cells exhibit reduced cell motility, decreased substrate attachment, and reduced invasion.

They continue:

The reduced expression of genes that are under the control of CpG island methylation is often reversed by treating the cells with the DNA methyl transferase inhibitor 5-Aza-29-deoxycytidine (5-Aza-dC). To assess the range and extent of miRNA expression under direct or indirect control of DNA methylation, we treated the melanoma cell line WM1552C (derived from a stage 3 malignant melanoma) with 5-Aza-dC and measured changes in miRNA gene expression using

miRNA microarrays (see Methods). Several miRNAs, including miR-34b, -489, -375, - 132, -142-3p, -200a, -145, -452, -21, -34c, -496, -let7e, -654, and -519b, were found to be up-regulated

They conclude:

During melanoma formation, the initial genetic or epigenetic changes are thought to precede additional mutations and further epigenetic changes that affect the function of several signaling pathways. Aberrant DNA methylation patterns at the 59 noncoding region of the INK4a gene was discovered in melanoma , which is consistent with the involvement of epigenetic factors in melanoma development or progression.

Similarly, epigenetic silencing of PTEN expression occurs in certain malignant melanomas with no detectable mutation in the PTEN gene.

While the impact on melanoma development of epigenetic changes in several protein-coding genes is appreciated, there have been few reports of the impact of epigenetic regulation of noncoding RNAs, such as miRNAs.

The epigenetic modification of miR-34b may serve as a useful biomarker for early melanoma detection in humans, and therefore, one could propose to develop a novel sensitive miR- 34b epigenetic biomarker assay to screen skin biopsies in melanoma patients. Including a panel of non-coding RNA epigenetic markers in to widely used pathological and genetic markers will be advantageous for both patients and pathologists.

An investigation of miR-34b regulation and associated CpG island methylation in a large group of melanoma patient samples, in comparison with samples of matched normal tissues or melanocytic nevi, is both relevant and timely. Mir-34 group of miRNAs are known to be useful therapeutic target for various cancers...

The PTEN control of cell proliferation is well known. However here it is shown that methylation can suppress PTEN without a genetic modification. Methylation is thus a powerful tool that surpasses genetic changes. Melanoma is an intriguing cancer because the effects of the environment are so well identified. Upon biopsy one can determine the extent of sun damage and ageing. Thus we can determine how much potential methylations effects are present as well.

1.1.1.8 Myelodysplastic Syndrome

Myelodysplastic Syndrome is an uncommon hematological cancer mostly caused by excess exposure to radiation, chemicals such as benzene, and insecticides. The specific genetic causes are still a work in progress. However, there are certain therapeutics which address some of the pathway aberrancies which characterize the disease, specifically hypermethylation.

As Taferri and Vardiman state:

According to the 2008 World Health Organization (WHO) classification system for hematologic cancers, the primary myelodysplastic syndromes are one of five major categories of myeloid

neoplasms. The main feature of myeloid neoplasms is stem-cell–derived clonal myelopoiesis with altered proliferation and differentiation. The phenotypic diversity of these neoplasms has been ascribed to different patterns of dysregulated signal transduction caused by transforming mutations that affect the hematopoietic stem cell. There is increasing evidence that haploinsufficiency, ***epigenetic changes****, and abnormalities in cytokines, the immune system, and bone marrow stroma all contribute to the development of the myelodysplastic syndromes.*

Thus MDS is both complex in presentation and complex in development. Melanoma and prostate cancer are more clearly characterized morphologically and generally in genetic development. The presentation may involve the white cells, red cells or platelets, or any combination thereof. It is often discovered as an incidental finding on a blood test with lowered amounts of one or several of the constituents. If it has progressed more it may also present in the bone biopsy with more than normal blasts, immature cells.

As DeVita et al state:

Myelodysplastic syndromes (MDSs) are a group of complex and heterogeneous clonal hematopoietic stem cell disorders whose defining characteristics are dysplasia of one or several hematopoietic cell lineages, hypercellular marrows, and blood cytopenias.

1 Although historically considered as a preleukemic state, most patients with MDS do not transform into an acute myeloid leukemia (AML), but will instead succumb to complications of persistent cytopenias. Indeed, the pathophysiology of MDS extends from immune-mediated mechanisms and excessive apoptosis resulting in marrow failure to arrest of maturation and proliferation resembling the mechanisms at play in AML.

2 The diverse pathophysiology of factors that contribute to the development of MDS is reflected in vast differences of patients' prognosis, which is increasingly recognized and reflected in the design of more elaborate systems of diagnosis, classification, and prognostication.

Let us begin with a simple set of statements regarding the micro RNA elements which are often seen at the heart of the disease. As Croce states:

Several of the miRNAs that have been described as suppressors have been found to be deleted or mutated in various human malignancies. For example, loss of miR-15a and miR-16-1 has also been observed in prostate cancer and multiple myeloma (TABLE 1). Members of the miR-29 family have been found to be deleted in a fraction of myelodysplastic syndrome (MDS) and acute myeloid leukaemia (AML) patients.

As Croce further states:

MicroRNAs as targets of epigenetic changes. The most studied epigenetic changes in cancer cells are the methylation of cytosines in the dinucleotide CpG in DNA62. Such 'methylable' sites, known as CpG islands, are preferentially located in the 5′ region (which consists of the promoter, 5′ uTR and exon 1) of many genes, are non-methylated in normal cells and are transcribed in the presence of the appropriate transcription factors. Methylation of the CpG

islands of tumour suppressors results in their silencing and contributes to malignant transformation.

As mentioned above, the expression of miRNAs can be affected by genetic changes, such as deletion, gene amplification and mutation, and by transcription factors. In addition, the expression of miRNAs can be affected by epigenetic changes, such as methylation of the CpG islands of their promoters. Saito et al. reported that miR-127 is silenced by promoter methylation in bladder tumours and that its expression could be restored by using hypomethylating agents such as azacitidine.

This miRNA targets BCL6, an oncogene that is involved in the development of diffuse large b cell lymphoma. Therefore, the silencing of miR-127 may lead to the overexpression of bCL6. Other investigators have described additional miRNAs that are silenced by methylation in various cancers and that can be reactivated by hypomethylating agents.

As Das and Singal state:

Hypermethylation is associated with many leukemias and other hematologic diseases. *Many genes, such as the calcitonin gene, p15INK4B, p21Cip1/Waf1, the ER gene, SDC4, MDR, and so on, were seen to be hypermethylated in a variety of hematologic cancers.*

The calcitonin gene and p15 were hypermethylated in 65% of myelodysplastic syndromes*, and it was found that p15 methylation at diagnosis was associated with lower survival and transformation to acute myeloid leukemia.*

Also acquisition of p15 methylation at a later date signaled disease progression. These may suggest the role of p15 as a marker of leukemic transformation. Acute myeloid leukemia demonstrated frequent hypermethylation of ER, MYOD1, PITX2, GPR37, and SDC4

Thus MDS is closely related to methylation, and in effect is caused by methylation. In addition as we show below its management is also performed through an understanding of methylation and managing that process.

1.1.1.9 Decitabine and MDS

Understanding the impact of methylation in MDS recent efforts have led to certain therapeutics which have been of help.

As Issa and Kantarjian state:

Two nucleoside inhibitors of DNA methylation, azacitidine and decitabine, are now standard of care for the treatment of the myelodysplastic syndrome, a deadly form of leukemia. These old drugs, developed as cytotoxic agents and nearly abandoned decades ago were resurrected by the renewed interest in DNA methylation.

They have now provided proof of principle for epigenetic therapy, the final chapter in the long saga to provide legitimacy to the field of epigenetics in cancer. But challenges remain; we don't understand precisely how or why the drugs work or stop working after an initial response. Extending these promising findings to solid tumors faces substantial hurdles from drug uptake to clinical trial design.

We do not know yet how to select patients for this therapy and how to move it from life extension to cure. The epigenetic potential of DNA methylation inhibitors may be limited by other epigenetic mechanisms that are also worth exploring as therapeutic targets. But the idea of stably changing gene expression in vivo has transformative potential in cancer therapy and beyond.

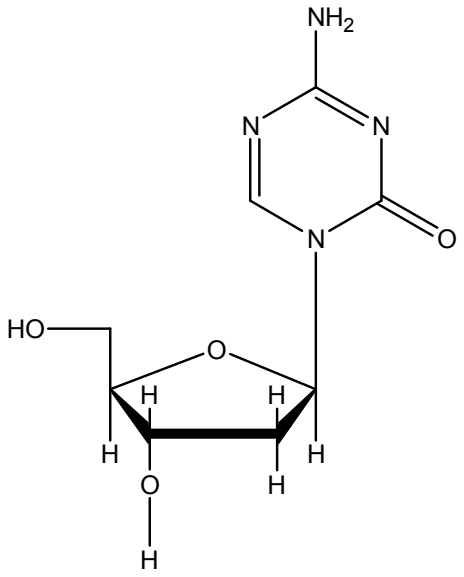
As Li has stated:

The strategies targeting DNA methylation. Epigenetic control of gene expression by DNA methylation has a great impact on cell proliferation and differentiation. Hypermethylation of promoter regions results in specific suppression of gene expression, including the expression of tumor suppressors, which could promote cancer development.

Conversely, demethylation of DNA may enhance cell apoptosis or reduce cell growth. This concept has been proven by a recently approved anticancer drug decitabine for the treatment of myelodysplastic syndrome. Decitabine (Dacogen; MGI Pharma) is a nucleoside analogue that inhibits DNA methylation.

It demethylates the p73 promoter and induces reexpression of p73, thus activating the caspase cascade and leading to leukemic myeloid cell death.26 DNA hypermethylation in tumor cells may be involved in resistance to interferon (INF)-induced apoptosis, and inhibition of DNA methylation may also enhance the therapeutic effect of INF. Treatment of cancer cells with specific DNA demethylating nucleoside analogue was shown to augment the effect of INF.

Now decitabine is shown below in detail. It is a cytosine derivative with several modifications. It functions in a manner similar to azacitidine. We have discussed that previously.



From Boumber et al we have the following regarding therapeutics for epigenetic drugs:

What Is Epigenetic Therapy? The understanding that epigenetic changes are prevalent in cancer and play a causative role in its biology has led to the development of new therapeutic approaches that target the epigenetic machinery. The first successful drugs developed as epigenetic agents were DNA methyltransferase inhibitors; these were followed by histone deacetylase inhibitors (HDIs).

Both classes of drugs aim at reversing gene silencing and demonstrate antitumor activity in vitro and in vivo. Several other classes of drugs have been developed that target various other components of the epigenetic machinery; one such class is the histone methyltransferases, with new drugs in this class currently in early preclinical development

The authors continue:

What Has Been Done? The inhibitors of DNA methylation used clinically are nucleoside analogues that get converted into deoxy-nucleotide-triphosphates (dNTPs) and become incorporated into DNA in place of cytosine during DNA replication. They trap all DNA methyltransferases and target them for degradation. At low doses these drugs do not inhibit proliferation; they reactivate gene expression and have shown clinical activity as anticancer agents. Azacitidine was the first hypomethylating agent approved by the FDA; its approval, in 2004, for the treatment of myelodysplastic disorders and leukemia, was followed by the approval, in 2006, of decitabine. Both drugs produce remissions or clinical improvements in more than 30% of patients treated. Features of responses have included the requirement for multiple cycles of therapy, slow response, and relatively few side effects. On the molecular level, demethylation, gene reactivation, and clonal elimination were observed in treated patients. The data in myelodysplastic syndrome (MDS) represent a proof-of-principle for epigenetic therapy for cancer, in particular in myeloid disorders.

From Boumber et al we have the following Table of many of the recent therapeutics:

Drug Class	Compound
DNMT Inhibitor	Azacitidine
	Decitabine
	S110
	CP-400
	Nanaomycin
HDAC Inhibitor	Vorinostat
	Romidepsin
	Panobinostat
	Valproic Acid
	Belinostat
HMT Inhibitor	Deazaneoplanocin
	Quinazoline
	Ellagic Acid
Histone demethylase inhibitor	Polyamine analogues
	Hydroxamate analogs
GAT inhibitor	Spermidinyl
	Hydrazinocurcumin
	Pyrazolone

As Stressman et al state:

Aberrant DNA methylation patterns play an important role in the pathogenesis of hematologic malignancies.

The DNA methyltransferase inhibitors azacytidine and decitabine have shown significant clinical benefits in the treatment of myelodysplastic syndrome (MDS), but their precise mode of action remains to be established. Both drugs have been shown the ability to deplete DNA methyltransferase enzymes and to induce DNA demethylation and epigenetic reprogramming in vitro. However, drug-induced methylation changes have remained poorly characterized in patients and therapy-related models.

We have now analyzed azacytidine-induced demethylation responses in myeloid leukemia cell lines. These cells showed remarkable differences in the drug-induced depletion of DNA methyltransferases that coincided with their demethylation responses. In agreement with these data, DNA methylation analysis of blood and bone marrow samples from MDS patients undergoing azacytidine therapy also revealed substantial differences in the epigenetic responses of individual patients.

Significant, transient demethylation could be observed in 3 of 6 patients and affected many hypermethylated loci in a complex pattern. Our results provide important proof-of-mechanism data for the demethylating activity of azacytidine in MDS patients and provide detailed insight into drug-induced demethylation responses.

1.1.1.10 Environmental and Genetic Causes and Factors

The main problem with MDS is that there is not clear genetic pathway and causal relationship. As DeVita et al state:

No etiologic factor is identified in most patients with MDS. MDS is more frequent in men than women by a factor of 1.8. It has been associated with smoking and hair dyes, exposure to agricultural and industrial toxins, drugs (e.g., chloramphenicol), and occupational exposures to stone and cereal dusts. MDS has been associated with exposure to ionizing radiation (atomic bomb survivors in Japan, decontamination workers following the Chernobyl nuclear plant accident) and chronic exposure to low-dose radiation (radiopharmaceuticals). Some inherited hematologic disorders (Fanconi anemia, dy-skeratosis congenita, Shwachman-Diamond syndrome, Diamond- Blackfan syndrome) are also associated with a higher risk of MDS.

Thus there is no clear causal factor or factors recognized at this time.

In a recent paper by Suzuki et al the authors discuss some of the causes of methylation and in turn cancers. They state:

Evidence now suggests that epigenetic abnormalities, particularly altered DNA methylation, play a crucial role in the development and progression of human gastrointestinal malignancies. Two distinct DNA methylation abnormalities are observed together in cancer.

One is an overall genome-wide reduction in DNA methylation (global hypomethylation) and the other is regional hypermethylation within the CpG islands of specific gene promoters. Global hypomethylation is believed to induce proto-oncogene activation and chromosomal instability, whereas regional hypermethylation is strongly associated with transcriptional silencing of tumor suppressor genes.

To date, genes involved in regulation of the cell cycle, DNA repair, growth signaling, angiogenesis, and apoptosis, are all known to be inactivated by hypermethylation. Recently developed techniques for detecting changes in DNA methylation have dramatically enhanced our understanding of the patterns of methylation that occur as cancers progress. One of the key contributors to aberrant methylation is aging, but other patterns of methylation are cancer-specific and detected only in a subset of tumors exhibiting the CpG island methylator phenotype (CIMP).

Although the cause of altered patterns of DNA methylation in cancer remains unknown, it is believed that epidemiological factors, notably dietary folate intake, might strongly influence DNA methylation patterns.

Recent studies further suggest that polymorphisms of genes involved in folate metabolism are causally related to the development of cancer.

5.8.9 Conclusion

This is a brief overview of methylation. We have attempted to describe it as one of many paths that lead to malignant cells.

1.1.1.11 Cause and Effect

As we have demonstrated there are no clear causal factors for the methylation we observe in many cancers. Although there are models for the effects of methylation on gene expression there is also not clear understanding of how much methylation is too much. It appears that once methylation begins it continues almost unabated until the expression of the affected gene is suppressed or otherwise modified.

1.1.1.12 Targeting: Activate or Suppress

The question is often; hypomethylated or hypermethylated, good or bad? Thus the therapy may also require that answer first. We can examine MDS and see that hypermethylation suppresses the genes controlling cell proliferation. No gene product and thus no control on the cell proliferation. It proliferates but poorly. Now can we just target the hypermethylation, it seems to function with decitabine. Are there other therapeutics such that an appropriate cocktail as used in many other treatments may be applied?

1.1.1.13 What Causes Methylation

The cause of methylation is a critical issue. We have argued that in melanoma it may be the result of UV radiation or even X Ray sources, with exposure as low as what one might see on a backscatter system deployed at airports. However there is still just speculation with limited data. We know that inflammation is a major source of such hypermethylation, and thus any inflammatory state would be a concern, for example as we often see in diabetics and alcoholics.

As Brower states:

Epigenetics has also provided clues that link environmental factors with cancerous genetic changes. Changes in methylation can be detected in the blood of cancer-free individuals who smoke and eat high-fat diets, and these changes have been shown to precede genetic mutations3. More recently, Karl Kelsey, a molecular epidemiologist at Brown University in Providence, Rhode Island, has uncovered independent associations between epigenetic patterns in breast cancer tumours, the tumour size, and alcohol consumption and folate intake.

A prime candidate at the interface of environment and genetics is chronic inflammation, which is known to precede the development of numerous types of precancerous lesions — and indeed certain cancers themselves, including oesophageal, liver and colon cancers. Inflammation has been linked with increased DNA methylation in otherwise healthy looking tissue. Issa calls chronic inflammation "a truly epigenetic phenomenon".

Long-term inflammation may result from infection with Helicobacter pylori or hepatitis C virus, or from autoimmune diseases such as ulcerative colitis (a form of inflammatory bowel disease). People with ulcerative colitis often develop colon cancer at a younger age — for example in their 50s — than the 60 to 70 year average age of onset.

Thus it still is clear that causal factors are speculative.

1.1.1.14 What can Prevent Methylation

Prevention of methylation will require clear causation. Reducing inflammation, perhaps various well accepted ways to do that may assist, but no clear path is laid out.

1.1.1.15 Can Damaging Methylation be Reversed

Having resulted in a hyper or hypo methylated state can it be reversed? That is a major clinical question. Decitabine is a typical example of a drug which appears to work.

As Brower states:

Drugs and dietary substances that alter epigenetic pathways are currently being tested. During his research on RCC, for example, Baylin and colleagues were able to reverse hypermethylation of the VHL gene with the drug 5-azacytidine. Trials of demethylating drugs as adjuvant treatments to prevent lung cancer recurrence are underway. If successful, prevention trials are the next logical step. "We need five- and ten-year survival data with current drugs to be sure there are no secondary effects before we give them to reasonably healthy people for prevention," says Issa. He sees a different source for the first wave of preventive medications. "I would bank on discovering more 'gentle' approaches to epigenetic manipulation for cancer prevention — be they natural products, existing drugs with a good safety records, or even vitamins or diet."

Thus many trials are underway but few solutions have been presented.

In conclusion, as Palii and Robertson state:

Epigenetic modifications are defined as heritable changes in gene expression occurring without alteration of underlying DNA sequence. A great deal of data has been accumulated showing the connection between neoplasia and dysregulated epigenetic processes.

Furthermore, cancer is now regarded as a multifaceted disease with a complex etiology, involving both mutational (genetic) and epigenetic alterations (such as DNA methylation and histone tail modifications).

DNA methylation status of biomarker genes is beginning to be employed for the assessment of patient samples and for prognostic purposes, and new techniques are being evaluated in the quest for yet unidentified TSGs as potential therapeutic targets DNA methylation represents a defense mechanism against selfish DNA elements, preserves the structural integrity of the

genome by "masking" repetitive sequences, and contributes to transcriptional repression and gene silencing.

Methylation targets CpG dinucleotides, which are generally underrepresented in mammalian genomes, except for promoter associated CpG islands, the only genomic regions in which CpG occurs at the expected frequency.

In normal cells, methylation is mostly present in pericentromeric regions, repetitive DNA, retro elements, and non-island CpGs, whereas methylation events in promoters and the body of genes have regulatory functions. Additionally, methylation is physiologic in the differentially methylated regions of imprinted genes where it ensures selective expression from a single parent of origin allele and in the inactive X chromosome in females.

CpG islands are generally protected against DNA methylation and therefore lack this modification in normal cells, although exceptions to this generalized rule have been found.

Thus methylation has both positive and negative effects. It is the serious and life threatening effects which we are concerned with. Palii and Robertson argue that Cancer is almost always epigenetic; methylation, miRNAs, and the like. However there are clear indications that pure genetic changes play a significant role as well.

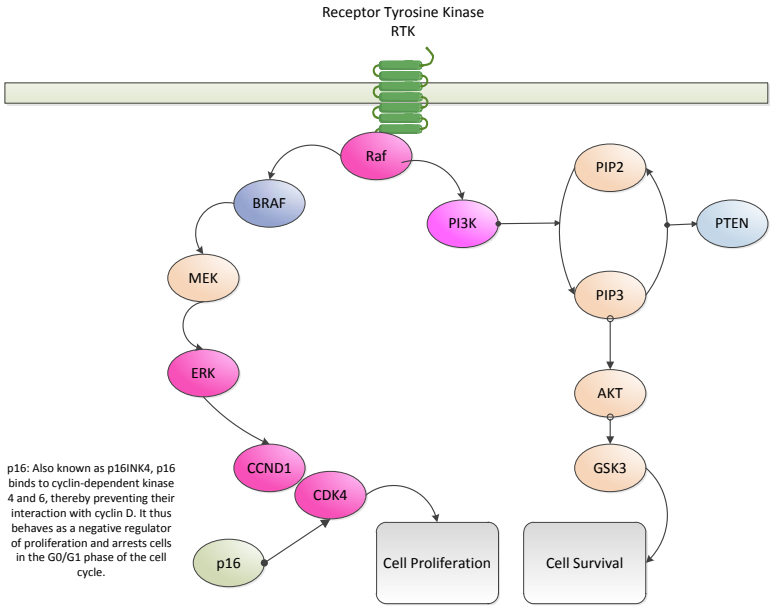
5.9 PATHWAYS AND IMMUNE RESPONSE

There has recently been several pathway control mechanisms developed and tested and also an immunological approach deemed to be somewhat effective. We examine them here and also use them as suggestive of what else may be accomplished.

The current methods focus on two areas: (i) controlling aberrant pathways and (ii) using the immune response to control aberrant cells. It should be noted that in both cases we are dealing with the paradigm of a single but multiplying yet identical cancer cell. There is no hypothesis as regards to a stem cell or to the fact that the cancer may be multi-clonal.

5.9.1 Aberrant Pathway Control

We now examine the aberrant pathway approach. First let us consider the pathways that control a single cell. We show them below:



The above shows two results; cell proliferation and cell survival. They are two characteristics of a cancer. Namely the cell replicates and it does so in an almost immortal manner. The changed cell then starts to take over where other functional cells have been and the result is an unstable and ultimately deadly takeover of the human. Thus the two pathways are but a few of the many we will discuss at length. Yet the key point is that in examining melanoma it has been discovered that there is a specific mutation in the B-RAF gene that activate the MEK pathway. Activating that pathway creates a situation where we have an uncontrolled growth.

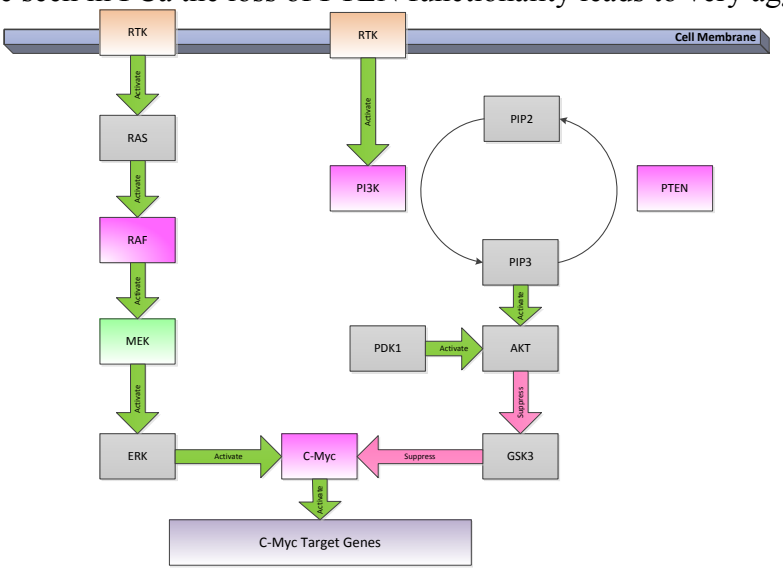
The growth factors activate the RTK kinase which activates the RAS which activates a B-RAF which overexpresses its product and this over-expression is what drives the proliferation pathway. It is this single gene and its protein expression which causes the problem in 60% of the cases.

The cell survival is often controlled by PTEN and it is the loss of PTEN which results in the cancer cell immortality. The PTEN loss is comparable to the same issue we have seen in prostate cancer.

Key to aberrant pathway control is a simple principle. Namely, we base the approach on the observed fact that certain pathway control elements have been changed as a result of a change in the underlying gene. We will show that in the current well known example of B-RAF that the underlying gene of B-RAF has been mutated and it the resulting B-RAF protein which has allowed the pathway to be turned on permanently. Thus the putative solution is to turn off the protein by targeting it with a drug which will pass the cell membrane and bind to the protein and inactivate it. A simple approach based upon an established fact. As we shall see there are two immediate issues: (i) only about 50% of the melanoma patients have the mutation, and (ii) the drug lasts for a relatively short time. It is similar to the effect that imatinib has on CML, a temporary regression and then a return.

As we shall see the possible solution may be multiple drug therapies targeting other pathway elements.

Now another way to view the pathways is shown below with the prominent role of c-Myc displayed at a common point. Note here we have the common surface kinases and the impact of B-RAF as well as PTEN. PTEN can modulate the limited up-regulation of B-RAF but only to a degree. As we have seen in PCa the loss of PTEN functionality leads to very aggressive forms.



The above also presents alternative control elements for possibly melanoma or frankly many other cancers. Specifically Smalley and Flaherty (2009) had suggested these pathway elements focusing on B-RAF, AKT and PI3K. One could also focus directly on the genes through a suppression mechanism but the technology for doing so is not yet available. Also there must be some specific targeting since we do not desire to target normal types of these products.

The control of aberrant pathways is conceptually simple.

1. Using a methodology such as microarrays, attempt to identify genes, or their expressions, which are present in the malignant cell. These are not unique and sometimes they are transient as well. The B-RAF identification is an example.

2. Develop a target molecule which can attach to and inactivate the aberrant gene or protein. In the current case of B-RAF they have deactivated the protein.

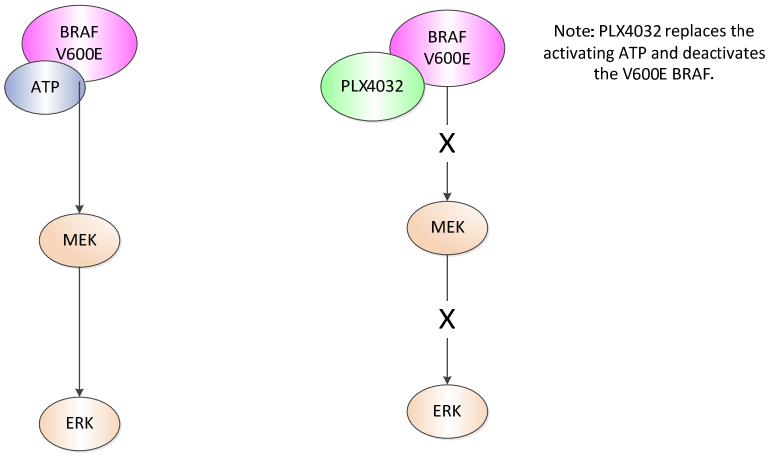
3. Test and use.

It may sound simple but the first step is potentially searching for a needle in a haystack and the second step can be as demanding. One may ask why not just block MEK or AKT just to stop everything. Assuming targets are possible the problem is it would do so for all cells and it would play havoc on the rest of the body. No blood cells, no hair, skin, and the like.

5.9.2 B-RAF control

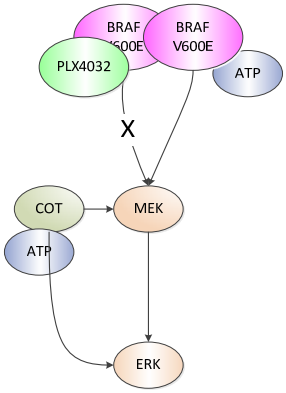
The most recent one is the control of a mutated B-RAF, a variant of the RAF pathway. It was observed that there was a mutation in the B-RAF gene so that what was produced was a different B-RAF called V600E which had excessive up-regulation in almost 50-60% of all metastatic melanomas. The identification of this product then allowed for its targeting and suppression as a means to reduce cell proliferation. The results have been reported recently by the work of Chapman et al (2011) and Flaherty et al (2010). A review by Smalley and Flaherty (2009) had made suggestions on controlling both the BRAF as well as the AKT pathway. We will discuss that later. Recent work by Poulikakos and Solit (2011) has also presented both BRAF and MEK control, trying to avoid the loss of efficacy we discuss here.

Specifically, a drug now called Vemurafenib, or PLX4032, binds to the ATP activation site on the B-RAF mutation V600E and as such it blocks the overexpression of this protein and reduces the flow downward which we have shown causes ultimately an up-regulation of proliferation.



Now we can also see that Vemurafenib can lose its effectiveness and there are several proposals for why this happens. We discuss a few here. From Solit and Rosen (2011) we show one of the possible ways in which resistance can occur. We discuss several of their conjectures in detail.

Below we depict the supposition from Solit and Rosen. Arguably this is what accounts for the mortality in the Kaplan Meir data they have from their trials.



From Solit and Rosen Fig 1: *"the overexpression of RAF1 or the activation of RAS as a result of RAS mutation or upstream activation of a receptor tyrosine kinase promotes:*

(i) the formation of RAF dimers. In cells expressing RAF dimers, binding of RAF inhibitors to one member of the dimer transactivates the other, nonbound member.

(ii) In such cells, PLX4032 does not inhibit MAP kinase signaling, which leads to drug resistance.

(iii) Alternatively, the overexpression of mitogen-activated protein kinase kinase kinase 8 (MAP3K8, or COT) results in RAF-independent activation of MEK and ERK and thus resistance to PLX4032.

(iv) The activation of upstream receptor tyrosine kinases may also cause resistance to PLX4032 by activating RAS, as well as by activating parallel signaling pathways, which results in diminished dependence of the cell on RAF signaling. PDGFRβ denotes platelet-derived growth factor receptor β, and RAS-GTP RAS in its active, GTP-bound state."

The paper by Solit and Rosen propose three reasons for loss of action of PLX4032:

(i) In melanomas with the BRAF V600E mutation, levels of activated RAS are too low to promote adequate formation of RAF dimers, and PLX4032 inhibits RAF activity and ERK signaling … This model is consistent with our observation that the introduction of mutant (activated) RAS into cells with mutant BRAF causes insensitivity of the ERK pathway to the drug. This model suggests that increases in RAF dimerization (because of RAS activation or increased RAF expression) will cause ERK signaling to become insensitive to PLX4032 …

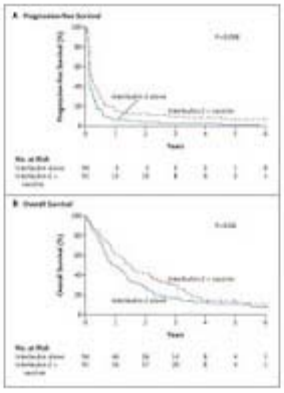
(ii) The findings of Johannessen et al. suggest another mechanism for the resistance of ERK signaling to RAF inhibition in cells driven by the BRAF V600E mutations. These investigators used a new technique — the introduction of a library of DNA constructs, each of which encodes a different kinase — into tumor cells with the BRAF V600E mutation to screen for kinases that confer resistance to RAF inhibition. Using this screen, they confirmed a previous finding: that overexpression of RAF1 confers resistance to RAF inhibition. [8] They further showed that the overexpression of mitogen-activated protein kinase kinase-kinase 8 (MAP3K8, or COT), which phosphorylates MEK in a RAF-independent manner, can also mediate resistance to RAF inhibitors…

(iii) a third basis for acquired resistance, one in which the activation of other pathways causes the tumor cell to become less dependent on ERK signaling. In these tumors, ERK activation remains sensitive to the RAF inhibitor. Specifically, they report that platelet derived growth factor receptor β (PDGFRβ), a receptor tyrosine kinase, is overexpressed in cellular models selected for RAF-inhibitor resistance in cell culture and in a subgroup of biopsy samples obtained from patients with progressing tumors. In the cell lines, PDGFRβ overexpression was associated with resistance to the anti-proliferative effects of the RAF inhibitor, despite continued inhibition of ERK signaling in the presence of the drug.

5.9.3 Immunological Techniques

Rosenberg has for decades been examining the use of the immune system to attack cancer cells and he has done a great deal of work specifically on melanoma. The second thrust of the recent advances has been along these lines and Rosenberg has also been a contributor.

The first recent report is by Schwartzentruber et al (2011, NEJM) wherein, along with Rosenberg, they have used a vaccination of a peptide which can recognize melanoma cells and then by increasing the T cells via an interleukin infusion they found that the result was improvement in survival of metastatic melanoma patients. We show the results below from the paper.



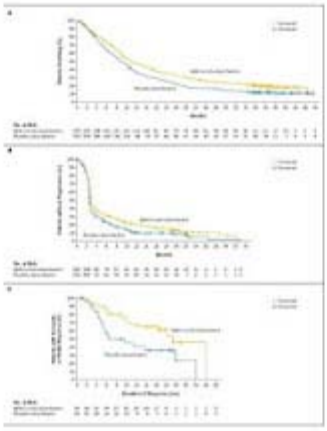
It should be noted that there is some improvement but still there is a very poor survival prognosis.

The second paper by Robert et al (2011 NEJM) uses another approach. They use a combination of a monoclonal antibody and a standard chemotherapeutic element. They state:

Ipilimumab, a fully human, IgG1 monoclonal antibody, blocks cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4), a negative regulator of T cells, and thereby augments T-cell activation and proliferation.

The second agent is dacarbazine. Decarbazine is a classic alkylating agent and has been used before with very limited results.

The data on survival is shown below:



Survival with the first approach after 36 months is about 38% and with the second approach it is about 45%. The interesting factor however with the second approach is the total remission in patients exhibiting total remission at the end of the study being almost 50%. Thus if total remission was exhibited it was sustained.

As the authors of the second study state:

Prolonged survival was noted among some patients who were followed for up to 4 years. In the ipilimumab–dacarbazine group, an estimated 28.5% of the patients were alive at 2 years, and an estimated 20.8% at 3 years, as compared with an estimated 17.9% and 12.2%, respectively, in the dacarbazine group.
One can seem to state that the second approach was more effective than the first.

Possibly combining the approaches will be more effective and the current understanding is that they intend to examine those paths.

5.9.4 Considerations

The current efforts clearly show some significant advancement. However there are several key issues which must be clarified:

1. Is melanoma like colon cancer as described by Vogelstein or do we have a somewhat random set of mutations depending on the location of the lesion. Namely is melanoma really a disparate set of different sub-cancers. Is there a clear genetic pathway, is there a gene that predisposes and if so how. The how is all too often the key question.

2. Where does the melanoma stem cell fit in this paradigm? Stem cells have a problem because if they exist and are of the primary concern then perhaps we are just eliminating the TIC cells and not the CSC.

3. What of the Harahan and Weinberg model of an interacting environment? Namely what about the influence of the other parts of the body including the immune system? This has been a Rosenberg issue for decades and Harahan and Weinberg make a strong case for its consideration.

4. Is it necessary to develop a data base of aberrant expressions of proteins?

5. What about dealing with the gene itself? Why just the protein.

6. How can we identify these cells from say cell surface markers. That would enhance the ability to expand our understanding of the histology down to the expression level.

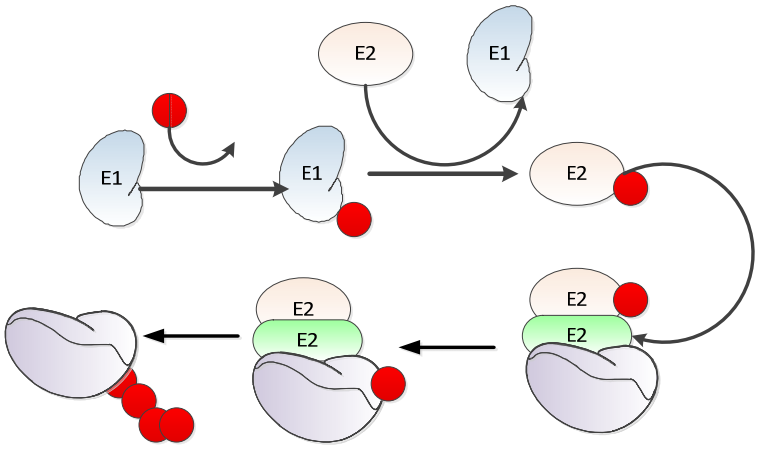
7. What genes have been changed and how? What was the change agent. We have argued elsewhere that it is radiation, ultraviolet and x-ray. But what of other factors. Where do the miRNAs fit, other epigenetic factors, methylation, and the like.

8. As with other cancers, there may be a sequence of changes, and is MIS, melanoma in situ, one of the steps. Is MIS akin to say HGPIN in prostate cancer or an adenoma in colon cancer?

There are many other issues which will evolve from this study. It represents a step in the forward direction but as has been seen each time we do this we see other new paths as unknown.

5.10 UBIQUITINATION

The normal destruction, consumption would be better, of cell proteins is performed by ubiquitin. The process is called Ubiquination. The process is explained graphically below.



Simply ubiquitin is released and proceeds to consume the protein to which it is attached. This is a normal process of cleaning up proteins and can be used in a cell to eliminate excess or irregular proteins. Specifically[96]:

[96] http://en.wikipedia.org/wiki/Ubiquitin

Ubiquitination is an enzymatic, protein post-translational modification (PTM) process in which the carboxylic acid of the terminal glycine from the di-glycine motif in the activated ubiquitin forms an amide bond to the epsilon amine of the lysine in the modified protein.

The process of marking a protein with ubiquitin (ubiquitination or ubiquitination) consists of a series of steps:

1. Activation of ubiquitin: Ubiquitin is activated in a two-step reaction by an E1 ubiquitin-activating enzyme in a process requiring ATP as an energy source. The initial step involves production of a ubiquitin-adenylate intermediate. The second step transfers ubiquitin to the E1 active site cysteine residue, with release of AMP. This step results in a thioester linkage between the C-terminal carboxyl group of ubiquitin and the E1 cysteine sulfhydryl group.

2. Transfer of ubiquitin from E1 to the active site cysteine of a ubiquitin-conjugating enzyme E2 via a trans(thio)esterification reaction. Mammalian genomes contain 30-40 UBCs.

3. The final step of the ubiquitination cascade creates an isopeptide bond between a lysine of the target protein and the C-terminal glycine of ubiquitin. In general, this step requires the activity of one of the hundreds of E3 ubiquitin-protein ligases (often termed simply ubiquitin ligase). E3 enzymes function as the substrate recognition modules of the system and are capable of interaction with both E2 and substrate.

In the ubiquitination cascade, E1 can bind with dozens of E2s, which can bind with hundreds of E3s in a hierarchical way. Other ubiquitin-like proteins (ULPs) are also modified via the E1–E2–E3 cascade.

5.11 CONCLUSION

This chapter is a key set of descriptors of the major pathways and their proteins. It does not speak of the dynamics nor does it detail the issues related to how failures occur. Further it details single cells only. That is a critical observation in modeling the whole in cancers.

What have we accomplished in the Chapter? Simply:

1. Identified the genes that control mitosis, proliferation, growth, and apoptosis.

2. Identified the surface receptors and activating gene products (ligands) which activate the internal pathways.

3. Identified some of the processes which result in the change to certain genes and gene products that result in dysfunction of gene pathways.

4. Identified the linkages of certain gene products and their control on pathways.

What have we failed to accomplish here? We have failed to explain a great deal. Simply:

1. The issue of gene products, proteins, and how they interact has not been addressed. Namely we assume that one molecule of PTEN can block one molecule in the PIP2/PIP3 cycle. Is just one required and if there are multiple PIP2/PIP3 molecules and multiple cycles, do we need multiple PTEN and thus how many.

2. The dynamics of the process was not considered. There are many models using these but they make substantial assumptions which may not be valid.

3. Reaction rates may not be realistic. Again many models of dynamics use classic high density assumptions regarding reactions and reaction rates. In a cell there are just a few proteins, and thus trying to use a large number of reaction rate models may not have merit.

4. In the many pathway models we have used, which is the best in that it reflects reality. There is the NCI/Nature Pathway Interaction Database, http://pid.nci.nih.gov/ , which demonstrates pathways to a substantial degree more than we have done here. Are these details flows useful? Are they too complex? How close to reality do we wish to get? We want to predict, but if we get too deep then the noise in the model may very well take over.

5.12 APPENDIX A: LIST OF GENES CLASSIFIED BY FUNCTION

We present here some 75 genes broken by category and then by gene. We rely upon the NIH data base for descriptions which are paraphrases of what we have presented in the text. One can see the expansiveness of this list compared to the 13 from Garraway and Chin as discussed earlier.

Element	*Type (Ligand, Receptor, Cell Surface, Pathway, Intra Nucleus, Transcription)*	*Function* http://www.ncbi.nlm.nih.gov/gene/
E cadherin	Cell Surface	This gene is a classical cadherin from the cadherin superfamily. The encoded protein is a calcium dependent cell-cell adhesion glycoprotein comprised of five extracellular cadherin repeats, a transmembrane region and a highly conserved cytoplasmic tail. Mutations in this gene are correlated with gastric, breast, colorectal, thyroid and ovarian cancer. Loss of function is thought to contribute to progression in cancer by increasing proliferation, invasion, and/or metastasis. The ectodomain of this protein mediates bacterial adhesion to mammalian cells and the cytoplasmic domain is required for internalization.
EGF	Ligand	This gene encodes a member of the epidermal growth factor superfamily. The encoded protein is synthesized as a large precursor molecule that is proteolytically cleaved to generate the 53-amino acid epidermal growth factor peptide. This protein acts a potent mitogenic factor that plays an important role in the growth, proliferation and differentiation of numerous cell types. This protein acts by binding the high affinity cell surface receptor, epidermal growth factor receptor. Defects in this gene are the cause of hypomagnesemia type 4. Dysregulation of this gene has been associated with the growth and progression of certain cancers.
Hedgehog	Ligand	This gene encodes a protein that is instrumental in patterning the early embryo. It has been implicated as the key inductive signal in patterning of the ventral neural tube, the anterior-posterior limb axis, and the ventral somites. Of three human proteins showing sequence and functional similarity to the sonic hedgehog protein of Drosophila, this protein is the most similar. The protein is made as a precursor that is autocatalytically cleaved; the N-terminal portion is soluble and contains the signalling activity while the C-terminal portion is involved in precursor processing. More importantly, the C-terminal product covalently attaches a cholesterol moiety to the N-terminal product, restricting the N-terminal product to the cell surface and preventing it from freely diffusing throughout the developing embryo. Defects in this protein or in its signalling pathway are a cause of holoprosencephaly (HPE), a disorder in which the developing forebrain fails to correctly separate into right and left hemispheres. HPE is manifested by facial deformities. It is also thought that mutations in this gene or in its signalling pathway may be responsible for VACTERL syndrome, which is characterized by vertebral defects, anal atresia, tracheoesophageal fistula with esophageal atresia, radial and renal dysplasia, cardiac anomalies, and limb abnormalities.

Her2	Ligand	This gene encodes a member of the epidermal growth factor (EGF) receptor family of receptor tyrosine kinases. This protein has no ligand binding domain of its own and therefore cannot bind growth factors. However, it does bind tightly to other ligand-bound EGF receptor family members to form a heterodimer, stabilizing ligand binding and enhancing kinase-mediated activation of downstream signalling pathways, such as those involving mitogen-activated protein kinase and phosphatidylinositol-3 kinase. Allelic variations at amino acid positions 654 and 655 of isoform a (positions 624 and 625 of isoform b) have been reported, with the most common allele, Ile654/Ile655, shown here. Amplification and/or overexpression of this gene have been reported in numerous cancers, including breast and ovarian tumors. Alternative splicing results in several additional transcript variants, some encoding different isoforms and others that have not been fully characterized.
HGF	Ligand	Hepatocyte growth factor regulates cell growth, cell motility, and morphogenesis by activating a tyrosine kinase signaling cascade after binding to the proto-oncogenic c-Met receptor. Hepatocyte growth factor is secreted by mesenchymal cells and acts as a multi-functional cytokine on cells of mainly epithelial origin. Its ability to stimulate mitogenesis, cell motility, and matrix invasion gives it a central role in angiogenesis, tumorogenesis, and tissue regeneration. It is secreted as a single inactive polypeptide and is cleaved by serine proteases into a 69-kDa alpha-chain and 34-kDa beta-chain. A disulfide bond between the alpha and beta chains produces the active, heterodimeric molecule. The protein belongs to the plasminogen subfamily of S1 peptidases but has no detectable protease activity.
IGFBP7	Ligand	This gene encodes a member of the insulin-like growth factor (IGF)-binding protein (IGFBP) family. IGFBPs bind IGFs with high affinity, and regulate IGF availability in body fluids and tissues and modulate IGF binding to its receptors. This protein binds IGF-I and IGF-II with relatively low affinity, and belongs to a subfamily of low-affinity IGFBPs. It also stimulates prostacyclin production and cell adhesion.

MAPK	Ligand	The protein encoded by this gene is a member of the MAP kinase family. MAP kinases, also known as extracellular signal-regulated kinases (ERKs), act as an integration point for multiple biochemical signals, and are involved in a wide variety of cellular processes such as proliferation, differentiation, transcription regulation and development. The activation of this kinase requires its phosphorylation by upstream kinases. Upon activation, this kinase translocates to the nucleus of the stimulated cells, where it phosphorylates nuclear targets. Two alternatively spliced transcript variants encoding the same protein, but differing in the UTRs, have been reported for this gene
PDGF	Ligand	The protein encoded by this gene is a member of the platelet-derived growth factor family. The four members of this family are mitogenic factors for cells of mesenchymal origin and are characterized by a motif of eight cysteines. This gene product can exist either as a homodimer (PDGF-BB) or as a heterodimer with the platelet-derived growth factor alpha polypeptide (PDGF-AB), where the dimers are connected by disulfide bonds. Mutations in this gene are associated with meningioma. Reciprocal translocations between chromosomes 22 and 7, at sites where this gene and that for COL1A1 are located, are associated with a particular type of skin tumor called dermatofibrosarcoma protuberans resulting from unregulated expression of growth factor. Two alternatively spliced transcript variants encoding different isoforms have been identified for this gene.
TGF	Ligand	This gene encodes a member of the transforming growth factor beta (TGFB) family of cytokines, which are multifunctional peptides that regulate proliferation, differentiation, adhesion, migration, and other functions in many cell types. Many cells have TGFB receptors, and the protein positively and negatively regulates many other growth factors. The secreted protein is cleaved into a latency-associated peptide (LAP) and a mature TGFB1 peptide, and is found in either a latent form composed of a TGFB1 homodimer, a LAP homodimer, and a latent TGFB1-binding protein, or in an active form composed of a TGFB1 homodimer. The mature peptide may also form heterodimers with other TGFB family members. This gene is frequently upregulated in tumor cells, and mutations in this gene result in Camurati-Engelmann disease

VEGF	Ligand	This gene is a member of the PDGF/VEGF growth factor family and encodes a protein that is often found as a disulfide linked homodimer. This protein is a glycosylated mitogen that specifically acts on endothelial cells and has various effects, including mediating increased vascular permeability, inducing angiogenesis, vasculogenesis and endothelial cell growth, promoting cell migration, and inhibiting apoptosis. Elevated levels of this protein are linked to POEMS syndrome, also known as Crow-Fukase syndrome. Mutations in this gene have been associated with proliferative and nonproliferative diabetic retinopathy. Alternatively spliced transcript variants, encoding either freely secreted or cell-associated isoforms, have been characterized. There is also evidence for the use of non-AUG (CUG) translation initiation sites upstream of, and in-frame with the first AUG, leading to additional isoforms
Wnt	Ligand	This gene is a member of the WNT gene family. The WNT gene family consists of structurally related genes which encode secreted signaling proteins. These proteins have been implicated in oncogenesis and in several developmental processes, including regulation of cell fate and patterning during embryogenesis. Alternatively spliced transcript variants have been identified for this gene.
ABL	Pathway	The *ABL1* proto-oncogene encodes a cytoplasmic and nuclear protein tyrosine kinase that has been implicated in processes of cell differentiation, cell division, cell adhesion, and stress response. Activity of ABL1 protein is negatively regulated by its SH3 domain, and deletion of the SH3 domain turns ABL1 into an oncogene.
AKT	Pathway	The serine-threonine protein kinase encoded by the AKT1 gene is catalytically inactive in serum-starved primary and immortalized fibroblasts. AKT1 and the related AKT2 are activated by platelet-derived growth factor. The activation is rapid and specific, and it is abrogated by mutations in the pleckstrin homology domain of AKT1. It was shown that the activation occurs through phosphatidylinositol 3-kinase.
AMPK	Pathway	The protein encoded by this gene is a regulatory subunit of the AMP-activated protein kinase (AMPK). AMPK is a heterotrimer consisting of an alpha catalytic subunit, and non-catalytic beta and gamma subunits. AMPK is an important energy-sensing enzyme that monitors cellular energy status. In response to cellular metabolic stresses, AMPK is activated, and thus phosphorylates and inactivates acetyl-CoA carboxylase (ACC) and beta-hydroxy beta-methylglutaryl-CoA reductase (HMGCR), key enzymes involved in regulating de novo biosynthesis of fatty acid and cholesterol. This subunit may be a positive regulator of AMPK activity. The myristoylation and phosphorylation of this subunit have been shown to affect the enzyme activity and cellular localization of AMPK. This subunit may also serve as an adaptor molecule mediating the association of the AMPK complex.
APC	Pathway	The activity of one protein in particular, beta-catenin is controlled by the APC protein (see: Wnt signaling pathway). Regulation of beta-catenin prevents genes that stimulate cell division from being turned on too often and prevents cell overgrowth.
ARF	Pathway	**p14ARF** is an alternate reading frame (ARF) product of the CDKN2A locus. Both p16INK4a and p14ARF are involved in cell cycle regulation. p14ARF inhibits mdm2, thus promoting p53, which promotes p21 activation, which then binds and inactivates certain cyclin-CDK complexes, which would otherwise promote transcription of genes that would carry the cell through the G_1/S checkpoint of the cell cycle. Loss of p14ARF by a homozygous mutation in the CDKN2A (INK4A) gene will lead to elevated levels in mdm2 and, therefore, loss of p53 function and cell cycle control.

BAD	Pathway	The **Bcl-2-associated death promoter** (**BAD**) protein is a pro-apoptotic member of the Bcl-2 gene family which is involved in initiating apoptosis. BAD is a member of the BH3-only family ,[1] a subfamily of the Bcl-2 family. It does not contain a C-terminal transmembrane domain for outer mitochondrial membrane and nuclear envelope targeting, unlike most other members of the Bcl-2 family.[2] After activation, it is able to form a heterodimer with anti-apoptotic proteins and prevent them from stopping apoptosis.
BMP	Pathway	The protein encoded by this gene belongs to the transforming growth factor-beta (TGFB) superfamily. The encoded protein acts as a disulfide-linked homodimer and induces bone and cartilage formation
Disheveled Dsh	Pathway	**Dishevelled** (Dsh) is a family of proteins involved in canonical and non-canonical Wnt signalling pathways. Dsh is a cytoplasmic phosphoprotein that acts directly downstream of frizzled receptors
ERK	Pathway	Ephrin receptors and their ligands, the ephrins, mediate numerous developmental processes, particularly in the nervous system. Based on their structures and sequence relationships, ephrins are divided into the ephrin-A (EFNA) class, which are anchored to the membrane by a glycosylphosphatidylinositol linkage, and the ephrin-B (EFNB) class, which are transmembrane proteins. The Eph family of receptors are divided into 2 groups based on the similarity of their extracellular domain sequences and their affinities for binding ephrin-A and ephrin-B ligands. Ephrin receptors make up the largest subgroup of the receptor tyrosine kinase (RTK) family.
GAS1	Pathway	Growth arrest-specific 1 plays a role in growth suppression. GAS1 blocks entry to S phase and prevents cycling of normal and transformed cells. Gas1 is a putative tumor suppressor gene.
GOLPH3	Pathway	The Golgi complex plays a key role in the sorting and modification of proteins exported from the endoplasmic reticulum. The protein encoded by this gene is a peripheral membrane protein of the Golgi stack and may have a regulatory role in Golgi trafficking. Several alternatively spliced transcript variants of this gene have been described, but the full-length nature of these variants has not been determined.
GSK-3β	Pathway	Glycogen synthase kinase-3 (GSK-3) is a proline-directed serine-threonine kinase that was initially identified as a phosphorylating and an inactivating agent of glycogen synthase. Two isoforms, alpha (GSK3A) and beta, show a high degree of amino acid homology.[1] GSK3B is involved in energy metabolism, neuronal cell development, and body pattern formation
LKB1	Pathway	This gene, which encodes a member of the serine/threonine kinase family, regulates cell polarity and functions as a tumor suppressor. Mutations in this gene have been associated with Peutz-Jeghers syndrome, an autosomal dominant disorder characterized by the growth of polyps in the gastrointestinal tract, pigmented macules on the skin and mouth, and other neoplasms. Alternate transcriptional splice variants of this gene have been observed but have not been thoroughly characterized.
MEK	Pathway	The protein encoded by this gene is a serine/threonine kinase and is part of some signal transduction cascades, including the ERK and JNK kinase pathways as well as the NF-kappa-B pathway. The encoded protein is activated by autophosphorylation and requires magnesium as a cofactor in phosphorylating other proteins

mTOR	Pathway	The mammalian target of rapamycin (mTOR) also known as mechanistic target of rapamycin or FK506 binding protein 12-rapamycin associated protein 1 (FRAP1) is a protein which in humans is encoded by the *FRAP1* gene.[1][2] mTOR is a serine/threonine protein kinase that regulates cell growth, cell proliferation, cell motility, cell survival, protein synthesis, and transcription.[3][4] mTOR belongs to the phosphatidylinositol 3-kinase-related kinase protein family.
NEDD9	Pathway	CRK-associated substrate-related protein; Cas scaffolding protein family member 2; Crk-associated substrate related; NEDD-9; cas-like docking; dJ49G10.2 (Enhancer of Filamentation 1 (HEF1)); dJ761I2.1 (enhancer of filamentation (HEF1)); enhancer of filamentation 1; neural precursor cell expressed developmentally down-regulated protein 9; p105; renal carcinoma antigen NY-REN-12
NF1	Pathway	NF1 encodes the protein neurofibromin, which appears to be a negative regulator of the ras signal transduction pathway. NF1 is found within the mammalian postsynapse, where it is known to bind to the NMDA receptor complex. It has been found to lead to deficits in learning, and it is suspected that this is a result of its regulation of the Ras pathway. It is known to regulate the GTPase HRAS, causing the hydrolyzation of GTP and thereby inactivating it
NRAS	Pathway	This is an N-ras oncogene encoding a membrane protein that shuttles between the Golgi apparatus and the plasma membrane. This shuttling is regulated through palmitoylation and depalmitoylation by the ZDHHC9-GOLGA7 complex. The encoded protein, which has intrinsic GTPase activity, is activated by a guanine nucleotide-exchange factor and inactivated by a GTPase activating protein. Mutations in this gene have been associated with somatic rectal cancer, follicular thyroid cancer, autoimmune lymphoproliferative syndrome, Noonan syndrome, and juvenile myelomonocytic leukemia.
p15	Pathway	CDKN2B: This gene lies adjacent to the tumor suppressor gene CDKN2A in a region that is frequently mutated and deleted in a wide variety of tumors. This gene encodes a cyclin-dependent kinase inhibitor, which forms a complex with CDK4 or CDK6, and prevents the activation of the CDK kinases, thus the encoded protein functions as a cell growth regulator that controls cell cycle G1 progression. The expression of this gene was found to be dramatically induced by TGF beta, which suggested its role in the TGF beta induced growth inhibition. Two alternatively spliced transcript variants of this gene, which encode distinct proteins, have been reported.
p16	Pathway	CDKN2A: This gene generates several transcript variants which differ in their first exons. At least three alternatively spliced variants encoding distinct proteins have been reported, two of which encode structurally related isoforms known to function as inhibitors of CDK4 kinase. The remaining transcript includes an alternate first exon located 20 Kb upstream of the remainder of the gene; this transcript contains an alternate open reading frame (ARF) that specifies a protein which is structurally unrelated to the products of the other variants. This ARF product functions as a stabilizer of the tumor suppressor protein p53 as it can interact with, and sequester, MDM1, a protein responsible for the degradation of p53. In spite of the structural and functional differences, the CDK inhibitor isoforms and the ARF product encoded by this gene, through the regulatory roles of CDK4 and p53 in cell cycle G1 progression, share a common functionality in cell cycle G1 control. This gene is frequently mutated or deleted in a wide variety of tumors, and is known to be an important tumor suppressor gene

p21	Pathway	CDKN1A:This gene encodes a potent cyclin-dependent kinase inhibitor. The encoded protein binds to and inhibits the activity of cyclin-CDK2 or -CDK4 complexes, and thus functions as a regulator of cell cycle progression at G1. The expression of this gene is tightly controlled by the tumor suppressor protein p53, through which this protein mediates the p53-dependent cell cycle G1 phase arrest in response to a variety of stress stimuli. This protein can interact with proliferating cell nuclear antigen (PCNA), a DNA polymerase accessory factor, and plays a regulatory role in S phase DNA replication and DNA damage repair. This protein was reported to be specifically cleaved by CASP3-like caspases, which thus leads to a dramatic activation of CDK2, and may be instrumental in the execution of apoptosis following caspase activation. Multiple alternatively spliced variants have been found for this gene.
p27	Pathway	The 26S proteasome is a multicatalytic proteinase complex with a highly ordered structure composed of 2 complexes, a 20S core and a 19S regulator. The 20S core is composed of 4 rings of 28 non-identical subunits; 2 rings are composed of 7 alpha subunits and 2 rings are composed of 7 beta subunits. The 19S regulator is composed of a base, which contains 6 ATPase subunits and 2 non-ATPase subunits, and a lid, which contains up to 10 non-ATPase subunits. Proteasomes are distributed throughout eukaryotic cells at a high concentration and cleave peptides in an ATP/ubiquitin-dependent process in a non-lysosomal pathway. An essential function of a modified proteasome, the immunoproteasome, is the processing of class I MHC peptides. This gene encodes a non-ATPase subunit of the 19S regulator. Three transcript variants encoding two different isoforms have been found for this gene.
p53	Pathway	This gene encodes tumor protein p53, which responds to diverse cellular stresses to regulate target genes that induce cell cycle arrest, apoptosis, senescence, DNA repair, or changes in metabolism. p53 protein is expressed at low level in normal cells and at a high level in a variety of transformed cell lines, where it's believed to contribute to transformation and malignancy. p53 is a DNA-binding protein containing transcription activation, DNA-binding, and oligomerization domains. It is postulated to bind to a p53-binding site and activate expression of downstream genes that inhibit growth and/or invasion, and thus function as a tumor suppressor. Mutants of p53 that frequently occur in a number of different human cancers fail to bind the consensus DNA binding site, and hence cause the loss of tumor suppressor activity. Alterations of this gene occur not only as somatic mutations in human malignancies, but also as germline mutations in some cancer-prone families with Li-Fraumeni syndrome. Multiple p53 variants due to alternative promoters and multiple alternative splicing have been found. These variants encode distinct isoforms, which can regulate p53 transcriptional activity

PAR1	Pathway	Progression of melanoma is dependent on cross-talk between tumor cells and the adjacent microenvironment. The thrombin receptor, protease-activated receptor-1 (PAR-1), plays a key role in exerting this function during melanoma progression. PAR-1 and its activating factors, which are expressed on tumor cells and the surrounding stroma, induce not only coagulation but also cell signaling, which promotes the metastatic phenotype. Several adhesion molecules, cytokines, growth factors, and proteases have recently been identified as downstream targets of PAR-1 and have been shown to modulate interactions between tumor cells and the microenvironment in the process of melanoma growth and metastasis. Inhibiting such interactions by targeting PAR-1 could potentially be a useful therapeutic modality for melanoma patients.
PI3K	Pathway	Phosphatidylinositol 3-kinase is composed of an 85 kDa regulatory subunit and a 110 kDa catalytic subunit. The protein encoded by this gene represents the catalytic subunit, which uses ATP to phosphorylate PtdIns, PtdIns4P and PtdIns(4,5)P2. This gene has been found to be oncogenic and has been implicated in cervical cancers.
PIP2 PIP3	Pathway	Phosphatidylinositol 4,5-bisphosphate (PIP2) is a minority phospholipid of the inner leaflet of plasma membranes. Many plasma membrane ion channels and ion transporters require PIP2 to function and can be turned off by signaling pathways that deplete PIP2. This review discusses the dependence of ion channels on phosphoinositides and considers possible mechanisms by which PIP2 and analogues regulate ion channel activity.
PREX2	Pathway	*PREX2* produces a protein that curtails the action of another protein called PTEN, which is involved in preventing cancer development.
PTEN	Pathway	This gene was identified as a tumor suppressor that is mutated in a large number of cancers at high frequency. The protein encoded this gene is a phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase. It contains a tensin like domain as well as a catalytic domain similar to that of the dual specificity protein tyrosine phosphatases. Unlike most of the protein tyrosine phosphatases, this protein preferentially dephosphorylates phosphoinositide substrates. It negatively regulates intracellular levels of phosphatidylinositol-3,4,5-trisphosphate in cells and functions as a tumor suppressor by negatively regulating AKT/PKB signaling pathway.
RAF	Pathway	This gene is the cellular homolog of viral raf gene (v-raf). The encoded protein is a MAP kinase kinase-kinase (MAP3K), which functions downstream of the Ras family of membrane associated GTPases to which it binds directly. Once activated, the cellular RAF1 protein can phosphorylate to activate the dual specificity protein kinases MEK1 and MEK2, which in turn phosphorylate to activate the serine/threonine specific protein kinases, ERK1 and ERK2. Activated ERKs are pleiotropic effectors of cell physiology and play an important role in the control of gene expression involved in the cell division cycle, apoptosis, cell differentiation and cell migration.

RAS	Pathway	The protein encoded by this gene is located in the cytoplasm and is part of the GAP1 family of GTPase-activating proteins. The gene product stimulates the GTPase activity of normal RAS p21 but not its oncogenic counterpart. Acting as a suppressor of RAS function, the protein enhances the weak intrinsic GTPase activity of RAS proteins resulting in the inactive GDP-bound form of RAS, thereby allowing control of cellular proliferation and differentiation. Mutations leading to changes in the binding sites of either protein are associated with basal cell carcinomas. Mutations also have been associated with hereditary capillary malformations (CM) with or without arteriovenous malformations (AVM) and Parkes Weber syndrome. Alternative splicing results in two isoforms where the shorter isoform, lacking the N-terminal hydrophobic region but retaining the same activity, appears to be abundantly expressed in placental but not adult tissues.
S6K1	Pathway	This gene encodes a member of the RSK (ribosomal S6 kinase) family of serine/threonine kinases. This kinase contains 2 non-identical kinase catalytic domains and phosphorylates several residues of the S6 ribosomal protein. The kinase activity of this protein leads to an increase in protein synthesis and cell proliferation. Amplification of the region of DNA encoding this gene and overexpression of this kinase are seen in some breast cancer cell lines. Alternate translational start sites have been described and alternate transcriptional splice variants have been observed but have not been thoroughly characterized.
SMAD4	Pathway	**SMADs** are intracellular proteins that transduce extracellular signals from transforming growth factor beta ligands to the nucleus where they activate downstream TGF-β gene transcription. The SMADs, which form a trimer of two receptor-regulated SMADs and one co-SMAD, act as transcription factors that regulate the expression of certain genes
SPOP	Pathway	**Speckle-type POZ protein** is a protein that in humans is encoded by the *SPOP* gene. This gene encodes a protein that may modulate the transcriptional repression activities of death-associated protein 6 (DAXX), which interacts with histone deacetylase, core histones, and other histone-associated proteins. In mouse, the encoded protein binds to the putative leucine zipper domain of macroH2A1.2, a variant H2A histone that is enriched on inactivated X chromosomes.

STRAD	Pathway	LKB1 specific adaptor protein STRAD. We use STRADA as per Boudeau et al. The protein encoded by this gene contains a STE20-like kinase domain, but lacks several residues that are critical for catalytic activity, so it is termed a 'pseudokinase'. The protein forms a heterotrimeric complex with serine/threonine kinase 11 (STK11, also known as LKB1) and the scaffolding protein calcium binding protein 39 (CAB39, also known as MO25). The protein activates STK11 leading to the phosphorylation of both proteins and excluding STK11 from the nucleus. The protein is necessary for STK11-induced G1 cell cycle arrest. A mutation in this gene has been shown to result in polyhydramnios, megalencephaly, and symptomatic epilepsy (PMSE) syndrome. Multiple transcript variants encoding different isoforms have been found for this gene.
TSC1	Pathway	This gene encodes a growth inhibitory protein thought to play a role in the stabilization of tuberin. Mutations in this gene have been associated with tuberous sclerosis. Alternative splicing results in multiple transcript variants.
TSC2	Pathway	Mutations in this gene lead to tuberous sclerosis complex. Its gene product is believed to be a tumor suppressor and is able to stimulate specific GTPases. The protein associates with hamartin in a cytosolic complex, possibly acting as a chaperone for hamartin. Alternative splicing results in multiple transcript variants encoding different isoforms.
β catenin	Pathway	The protein encoded by this gene is part of a complex of proteins that constitute adherens junctions (AJs). AJs are necessary for the creation and maintenance of epithelial cell layers by regulating cell growth and adhesion between cells. The encoded protein also anchors the actin cytoskeleton and may be responsible for transmitting the contact inhibition signal that causes cells to stop dividing once the epithelial sheet is complete. Finally, this protein binds to the product of the APC gene, which is mutated in adenomatous polyposis of the colon. Mutations in this gene are a cause of colorectal cancer (CRC), pilomatrixoma (PTR), medulloblastoma (MDB), and ovarian cancer.
EGFR	Receptor	The protein encoded by this gene is a transmembrane glycoprotein that is a member of the protein kinase superfamily. This protein is a receptor for members of the epidermal growth factor family. EGFR is a cell surface protein that binds to epidermal growth factor. Binding of the protein to a ligand induces receptor dimerization and tyrosine autophosphorylation and leads to cell proliferation. Mutations in this gene are associated with lung cancer. Multiple alternatively spliced transcript variants that encode different protein isoforms have been found for this gene.
ERBB4	Receptor	**Receptor tyrosine-protein kinase erbB-4** is an enzyme that in humans is encoded by the *ERBB4* gene.[1][2] Alternatively spliced variants that encode different protein isoforms have been described; however, not all variants have been fully characterized.[3] Receptor tyrosine-protein kinase erbB-4 is a receptor tyrosine kinase that is a member of the epidermal growth factor receptor subfamily. ERBB4 is a single-pass type I transmembrane protein with multiple furin-like cysteine rich domains, a tyrosine kinase domain, a phosphotidylinositol-3 kinase binding site and a PDZ domain binding motif. The protein binds to and is activated by neuregulins-2 and -3, heparin-binding EGF-like growth factor and betacellulin.

FGFR	Receptor	This gene encodes a member of the fibroblast growth factor receptor (FGFR) family, with its amino acid sequence being highly conserved between members and among divergent species. FGFR family members differ from one another in their ligand affinities and tissue distribution. A full-length representative protein would consist of an extracellular region, composed of three immunoglobulin-like domains, a single hydrophobic membrane-spanning segment and a cytoplasmic tyrosine kinase domain. The extracellular portion of the protein interacts with fibroblast growth factors, setting in motion a cascade of downstream signals, ultimately influencing mitogenesis and differentiation. This particular family member binds acidic and basic fibroblast growth hormone and plays a role in bone development and maintenance.
Frizzled	Receptor	Wnt binding site. This gene is a member of the frizzled gene family. Members of this family encode seven-transmembrane domain proteins that are receptors for the Wingless type MMTV integration site family of signaling proteins. Most frizzled receptors are coupled to the beta-catenin canonical signaling pathway. This protein may play a role as a positive regulator of the Wingless type MMTV integration site signaling pathway. A transcript variant retaining intronic sequence and encoding a shorter isoform has been described, however, its expression is not supported by other experimental evidence.
GR	Receptor	Growth receptor. This gene encodes glucocorticoid receptor, which can function both as a transcription factor that binds to glucocorticoid response elements in the promoters of glucocorticoid responsive genes to activate their transcription, and as a regulator of other transcription factors. This receptor is typically found in the cytoplasm, but upon ligand binding, is transported into the nucleus. It is involved in inflammatory responses, cellular proliferation, and differentiation in target tissues. Mutations in this gene are associated with generalized glucocorticoid resistance. Alternative splicing of this gene results in transcript variants encoding either the same or different isoforms. Additional isoforms resulting from the use of alternate in-frame translation initiation sites have also been described, and shown to be functional, displaying diverse cytoplasm-to-nucleus trafficking patterns and distinct transcriptional activities
IR	Receptor	Insulin receptor. After removal of the precursor signal peptide, the insulin receptor precursor is post-translationally cleaved into two chains (alpha and beta) that are covalently linked. Binding of insulin to the insulin receptor (INSR) stimulates glucose uptake. Two transcript variants encoding different isoforms have been found for this gene.
KIT	Receptor	This gene encodes the human homolog of the proto-oncogene c-kit. C-kit was first identified as the cellular homolog of the feline sarcoma viral oncogene v-kit. This protein is a type 3 transmembrane receptor for MGF (mast cell growth factor, also known as stem cell factor). Mutations in this gene are associated with gastrointestinal stromal tumors, mast cell disease, acute myelogenous lukemia, and piebaldism.

Notched	Receptor	This gene encodes a member of the Notch family. Members of this Type 1 transmembrane protein family share structural characteristics including an extracellular domain consisting of multiple epidermal growth factor-like (EGF) repeats, and an intracellular domain consisting of multiple, different domain types. Notch family members play a role in a variety of developmental processes by controlling cell fate decisions. The Notch signaling network is an evolutionarily conserved intercellular signaling pathway which regulates interactions between physically adjacent cells. In Drosophilia, notch interaction with its cell-bound ligands (delta, serrate) establishes an intercellular signaling pathway that plays a key role in development. Homologues of the notch-ligands have also been identified in human, but precise interactions between these ligands and the human notch homologues remain to be determined. This protein is cleaved in the trans-Golgi network, and presented on the cell surface as a heterodimer. This protein functions as a receptor for membrane bound ligands, and may play multiple roles during development.
Patched	Receptor	This gene encodes a member of the patched gene family. The encoded protein is the receptor for sonic hedgehog, a secreted molecule implicated in the formation of embryonic structures and in tumorigenesis, as well as the desert hedgehog and indian hedgehog proteins. This gene functions as a tumor suppressor. Mutations of this gene have been associated with basal cell nevus syndrome, esophageal squamous cell carcinoma, trichoepitheliomas, transitional cell carcinomas of the bladder, as well as holoprosencephaly. Alternative splicing results in multiple transcript variants encoding different isoforms.
Smoothened	Receptor	The protein encoded by this gene is a G protein-coupled receptor that interacts with the patched protein, a receptor for hedgehog proteins. The encoded protein tranduces signals to other proteins after activation by a hedgehog protein/patched protein complex
4EBP1	Transcription	This gene encodes one member of a family of translation repressor proteins. The protein directly interacts with eukaryotic translation initiation factor 4E (eIF4E), which is a limiting component of the multisubunit complex that recruits 40S ribosomal subunits to the 5' end of mRNAs. Interaction of this protein with eIF4E inhibits complex assembly and represses translation. This protein is phosphorylated in response to various signals including UV irradiation and insulin signaling, resulting in its dissociation from eIF4E and activation of mRNA translation.
CCND1	Transcription	The protein encoded by this gene belongs to the highly conserved cyclin family, whose members are characterized by a dramatic periodicity in protein abundance throughout the cell cycle. Cyclins function as regulators of CDK kinases.
CDK4	Transcription	The protein encoded by this gene is a member of the Ser/Thr protein kinase family. This protein is highly similar to the gene products of S. cerevisiae cdc28 and S. pombe cdc2. It is a catalytic subunit of the protein kinase complex that is important for cell cycle G1 phase progression. The activity of this kinase is restricted to the G1-S phase, which is controlled by the regulatory subunits D-type cyclins and CDK inhibitor p16(INK4a).

CREB	Transcription	This gene encodes a transcription factor that is a member of the leucine zipper family of DNA binding proteins. This protein binds as a homodimer to the cAMP-responsive element, an octameric palindrome. The protein is phosphorylated by several protein kinases, and induces transcription of genes in response to hormonal stimulation of the cAMP pathway. Alternate splicing of this gene results in two transcript variants encoding different isoforms.
eIF4E	Transcription	The protein encoded by this gene is a component of the eukaryotic translation initiation factor 4F complex, which recognizes the 7-methylguanosine cap structure at the 5' end of cellular mRNAs. The encoded protein aids in translation initiation by recruiting ribosomes to the mRNA. Association of this protein with the 4F complex is the rate-limiting step in translation initiation. Three transcript variants encoding different isoforms have been found for this gene.
ETV1	Transcription	**ETS translocation variant 1** is a protein that in humans is encoded by the *ETV1* gene. This gene encodes a member of the ETS (E twenty-six) family of transcription factors. The ETS proteins regulate many target genes that modulate biological processes like cell growth, angiogenesis, migration, proliferation and differentiation. All ETS proteins contain an ETS DNA-binding domain that binds to DNA sequences containing the consensus 5'-CGGA[AT]-3'. The protein encoded by this gene contains a conserved short acidic transactivation domain (TAD) in the N-terminal region, in addition to the ETS DNA-binding domain in the C-terminal region.
FOS	Transcription	**c-Jun** is the name of a gene and protein that, in combination with c-Fos, forms the AP-1 early response transcription factor. It was first identified as the Fos-binding protein p39 and only later rediscovered as the product of the c-jun gene. It is activated through double phosphorylation by the JNK pathway but has also a phosphorylation-independent function. c-Jun knockout is lethal, but transgenic animals with a mutated c-Jun that cannot be phosphorylated (termed c-JunAA) can survive.
GLI2	Transcription	GLI2 belongs to the C2H2-type zinc finger protein subclass of the Gli family. Members of this subclass are characterized as transcription factors which bind DNA through zinc finger motifs

HIF	Transcription	This gene encodes the alpha subunit of transcription factor hypoxia-inducible factor-1 (HIF-1), which is a heterodimer composed of an alpha and a beta subunit. HIF-1 functions as a master regulator of cellular and systemic homeostatic response to hypoxia by activating transcription of many genes, including those involved in energy metabolism, angiogenesis, apoptosis, and other genes whose protein products increase oxygen delivery or facilitate metabolic adaptation to hypoxia. HIF-1 thus plays an essential role in embryonic vascularization, tumor angiogenesis and pathophysiology of ischemic disease. Alternatively spliced transcript variants encoding different isoforms have been identified for this gene.
INK4A	Transcription	This gene generates several transcript variants which differ in their first exons. At least three alternatively spliced variants encoding distinct proteins have been reported, two of which encode structurally related isoforms known to function as inhibitors of CDK4 kinase. The remaining transcript includes an alternate first exon located 20 Kb upstream of the remainder of the gene; this transcript contains an alternate open reading frame (ARF) that specifies a protein which is structurally unrelated to the products of the other variants. This ARF product functions as a stabilizer of the tumor suppressor protein p53 as it can interact with, and sequester, MDM1, a protein responsible for the degradation of p53. In spite of the structural and functional differences, the CDK inhibitor isoforms and the ARF product encoded by this gene, through the regulatory roles of CDK4 and p53 in cell cycle G1 progression, share a common functionality in cell cycle G1 control. This gene is frequently mutated or deleted in a wide variety of tumors, and is known to be an important tumor suppressor gene.
JUN	Transcription	See FOS
LEF	Transcription	This gene encodes a transcription factor belonging to a family of proteins that share homology with the high mobility group protein-1. The protein encoded by this gene can bind to a functionally important site in the T-cell receptor-alpha enhancer, thereby conferring maximal enhancer activity. This transcription factor is involved in the Wnt signaling pathway, and it may function in hair cell differentiation and follicle morphogenesis. Mutations in this gene have been found in somatic sebaceous tumors. This gene has also been linked to other cancers, including androgen-independent prostate cancer.
MITF	Transcription	Microphthalmia-associated transcription factor (MITF) is a basic helix-loop-helix leucine zipper transcription factor involved in melanocyte and osteoclast development
MYC	Transcription	The protein encoded by this gene is a multifunctional, nuclear phosphoprotein that plays a role in cell cycle progression, apoptosis and cellular transformation. It functions as a transcription factor that regulates transcription of specific target genes. Mutations, overexpression, rearrangement and translocation of this gene have been associated with a variety of hematopoietic tumors, leukemias and lymphomas, including Burkitt lymphoma. There is evidence to show that alternative translation initiations from an upstream, in-frame non-AUG (CUG) and a downstream AUG start site result in the production of two isoforms with distinct N-termini. The synthesis of non-AUG initiated protein is suppressed in Burkitt's lymphomas, suggesting its importance in the normal function of this gene.
NF-κB	Transcription	**NF-κB** (**nuclear factor kappa-light-chain-enhancer of activated B cells**) is a protein complex that controls the transcription of DNA. NF-κB is found in almost all animal cell types and is involved in cellular responses to stimuli such as stress, cytokines, free radicals, ultraviolet irradiation, oxidized LDL, and bacterial or viral antigens

TCF	Transcription	The protein encoded by this gene is a nuclear transcription factor which binds DNA as a homodimer. The encoded protein controls the expression of several genes, including hepatocyte nuclear factor 1 alpha, a transcription factor which regulates the expression of several hepatic genes. This gene may play a role in development of the liver, kidney, and intestines. Mutations in this gene have been associated with monogenic autosomal dominant non-insulin-dependent diabetes mellitus type I. Alternative splicing of this gene results in multiple transcript variants encoding several different isoforms.
TCF	Transcription	The protein encoded by this gene is a nuclear transcription factor which binds DNA as a homodimer. The encoded protein controls the expression of several genes, including hepatocyte nuclear factor 1 alpha, a transcription factor which regulates the expression of several hepatic genes. This gene may play a role in development of the liver, kidney, and intestines. Mutations in this gene have been associated with monogenic autosomal dominant non-insulin-dependent diabetes mellitus type I. Alternative splicing of this gene results in multiple transcript variants encoding several different isoforms.
TOR	Transcription	The protein encoded by this gene is a DNA-binding transcription factor and is a member of the NR1 subfamily of nuclear hormone receptors. The specific functions of this protein are not known; however, studies of a similar gene in mice have shown that this gene may be essential for lymphoid organogenesis and may play an important regulatory role in thymopoiesis. In addition, studies in mice suggest that the protein encoded by this gene may inhibit the expression of Fas ligand and IL2. Two transcript variants encoding different isoforms have been found for this gene.

5.13 APPENDIX B: PH GENES AND LOCATIONS

The following Table is a list of the gene specifically related to the PH analysis. It provides gene location as well.

Gene[97]	*Location*	*Function*
LKB1 also STK11	19p13.3	This gene, which encodes a member of the serine/threonine kinase family, regulates cell polarity and functions as a tumor suppressor. Mutations in this gene have been associated with Peutz-Jeghers syndrome, an autosomal dominant disorder characterized by the growth of polyps in the gastrointestinal tract, pigmented macules on the skin and mouth, and other neoplasms. Alternate transcriptional splice variants of this gene have been observed but have not been thoroughly characterized.
STRAD	17q23.3	LKB1 specific adaptor protein STRAD. We use STRADA as per Boudeau et al. The protein encoded by this gene contains a STE20-like kinase domain, but lacks several residues that are critical for catalytic activity, so it is termed a 'pseudokinase'. The protein forms a heterotrimeric complex with serine/threonine kinase 11 (STK11, also known as LKB1) and the scaffolding protein calcium binding protein 39 (CAB39, also known as MO25). The protein activates STK11 leading to the phosphorylation of both proteins and excluding STK11 from the nucleus. The protein is necessary for STK11-induced G1 cell cycle arrest. A mutation in this gene has been shown to result in polyhydramnios, megalencephaly, and symptomatic epilepsy (PMSE) syndrome. Multiple transcript variants encoding different isoforms have been found for this gene.
PAR1	15q11.2	Progression of melanoma is dependent on cross-talk between tumor cells and the adjacent microenvironment. The thrombin receptor, protease-activated receptor-1 (PAR-1), plays a key role in exerting this function during melanoma progression. PAR-1 and its activating factors, which are expressed on tumor cells and the surrounding stroma, induce not only coagulation but also cell signaling, which promotes the metastatic phenotype. Several adhesion molecules, cytokines, growth factors, and proteases have recently been identified as downstream targets of PAR-1 and have been shown to modulate interactions between tumor cells and the microenvironment in the process of melanoma growth and metastasis. Inhibiting such interactions by targeting PAR-1 could potentially be a useful therapeutic modality for melanoma patients.
MTOR	1p36.2	The protein encoded by this gene belongs to a family of phosphatidylinositol kinase-related kinases. These kinases mediate cellular responses to stresses such as DNA damage and nutrient deprivation. This protein acts as the target for the cell-cycle arrest and immunosuppressive effects of the FKBP12-rapamycin complex.
4EBP1	8p12	This gene encodes one member of a family of translation repressor proteins. The protein directly interacts with eukaryotic translation initiation factor 4E (eIF4E), which is a limiting component of the multisubunit complex that recruits 40S ribosomal subunits to the 5' end of mRNAs. Interaction of this protein with eIF4E inhibits complex assembly and represses translation. This protein is phosphorylated in response to various signals including UV irradiation and insulin signaling, resulting in its dissociation from eIF4E and activation of mRNA translation.

[97] http://www.ncbi.nlm.nih.gov/gene/6794 we use this source for gene descriptions.

Gene[97]	*Location*	*Function*
eIF4E	4q21-25	The protein encoded by this gene is a component of the eukaryotic translation initiation factor 4F complex, which recognizes the 7-methylguanosine cap structure at the 5' end of cellular mRNAs. The encoded protein aids in translation initiation by recruiting ribosomes to the mRNA. Association of this protein with the 4F complex is the rate-limiting step in translation initiation. Three transcript variants encoding different isoforms have been found for this gene.
S6K1	17q23.1	This gene encodes a member of the RSK (ribosomal S6 kinase) family of serine/threonine kinases. This kinase contains 2 non-identical kinase catalytic domains and phosphorylates several residues of the S6 ribosomal protein. The kinase activity of this protein leads to an increase in protein synthesis and cell proliferation. Amplification of the region of DNA encoding this gene and overexpression of this kinase are seen in some breast cancer cell lines. Alternate translational start sites have been described and alternate transcriptional splice variants have been observed but have not been thoroughly characterized.
AMPK	14q24.1	The protein encoded by this gene is a regulatory subunit of the AMP-activated protein kinase (AMPK). AMPK is a heterotrimer consisting of an alpha catalytic subunit, and non-catalytic beta and gamma subunits. AMPK is an important energy-sensing enzyme that monitors cellular energy status. In response to cellular metabolic stresses, AMPK is activated, and thus phosphorylates and inactivates acetyl-CoA carboxylase (ACC) and beta-hydroxy beta-methylglutaryl-CoA reductase (HMGCR), key enzymes involved in regulating de novo biosynthesis of fatty acid and cholesterol. This subunit may be a positive regulator of AMPK activity. The myristoylation and phosphorylation of this subunit have been shown to affect the enzyme activity and cellular localization of AMPK. This subunit may also serve as an adaptor molecule mediating the association of the AMPK complex.
Akt	14q32.32	The serine-threonine protein kinase encoded by the AKT1 gene is catalytically inactive in serum-starved primary and immortalized fibroblasts. AKT1 and the related AKT2 are activated by platelet-derived growth factor. The activation is rapid and specific, and it is abrogated by mutations in the pleckstrin homology domain of AKT1. It was shown that the activation occurs through phosphatidylinositol 3-kinase. In the developing nervous system AKT is a critical mediator of growth factor-induced neuronal survival. Survival factors can suppress apoptosis in a transcription-independent manner by activating the serine/threonine kinase AKT1, which then phosphorylates and inactivates components of the apoptotic machinery. Mutations in this gene have been associated with the Proteus syndrome. Multiple alternatively spliced transcript variants have been found for this gene.
TSC1	9q34	This gene encodes a growth inhibitory protein thought to play a role in the stabilization of tuberin. Mutations in this gene have been associated with tuberous sclerosis. Alternative splicing results in multiple transcript variants.
TSC2	16p13.3	Mutations in this gene lead to tuberous sclerosis complex. Its gene product is believed to be a tumor suppressor and is able to stimulate specific GTPases. The protein associates with hamartin in a cytosolic complex, possibly acting as a chaperone for hamartin. Alternative splicing results in multiple transcript variants encoding different isoforms.

Gene[97]	*Location*	*Function*
HIF	14q23.2	This gene encodes the alpha subunit of transcription factor hypoxia-inducible factor-1 (HIF-1), which is a heterodimer composed of an alpha and a beta subunit. HIF-1 functions as a master regulator of cellular and systemic homeostatic response to hypoxia by activating transcription of many genes, including those involved in energy metabolism, angiogenesis, apoptosis, and other genes whose protein products increase oxygen delivery or facilitate metabolic adaptation to hypoxia. HIF-1 thus plays an essential role in embryonic vascularization, tumor angiogenesis and pathophysiology of ischemic disease. Alternatively spliced transcript variants encoding different isoforms have been identified for this gene.
VEGF	6p12	This gene is a member of the PDGF/VEGF growth factor family and encodes a protein that is often found as a disulfide linked homodimer. This protein is a glycosylated mitogen that specifically acts on endothelial cells and has various effects, including mediating increased vascular permeability, inducing angiogenesis, vasculogenesis and endothelial cell growth, promoting cell migration, and inhibiting apoptosis. Elevated levels of this protein are linked to POEMS syndrome, also known as Crow-Fukase syndrome. Mutations in this gene have been associated with proliferative and nonproliferative diabetic retinopathy. Alternatively spliced transcript variants, encoding either freely secreted or cell-associated isoforms, have been characterized. There is also evidence for the use of non-AUG (CUG) translation initiation sites upstream of, and in-frame with the first AUG, leading to additional isoforms
TGF	19q13.1	This gene encodes a member of the transforming growth factor beta (TGFB) family of cytokines, which are multifunctional peptides that regulate proliferation, differentiation, adhesion, migration, and other functions in many cell types. Many cells have TGFB receptors, and the protein positively and negatively regulates many other growth factors. The secreted protein is cleaved into a latency-associated peptide (LAP) and a mature TGFB1 peptide, and is found in either a latent form composed of a TGFB1 homodimer, a LAP homodimer, and a latent TGFB1-binding protein, or in an active form composed of a TGFB1 homodimer. The mature peptide may also form heterodimers with other TGFB family members. This gene is frequently upregulated in tumor cells, and mutations in this gene result in Camurati-Engelmann disease.
PDGF	22q13.1	The protein encoded by this gene is a member of the platelet-derived growth factor family. The four members of this family are mitogenic factors for cells of mesenchymal origin and are characterized by a motif of eight cysteines. This gene product can exist either as a homodimer (PDGF-BB) or as a heterodimer with the platelet-derived growth factor alpha polypeptide (PDGF-AB), where the dimers are connected by disulfide bonds. Mutations in this gene are associated with meningioma. Reciprocal translocations between chromosomes 22 and 7, at sites where this gene and that for COL1A1 are located, are associated with a particular type of skin tumor called dermatofibrosarcoma protuberans resulting from unregulated expression of growth factor. Two alternatively spliced transcript variants encoding different isoforms have been identified for this gene.
PIP2		Phosphatidylinositol 4,5-bisphosphate (PIP2) is a minority phospholipid of the inner leaflet of plasma membranes. Many plasma membrane ion channels and ion transporters require PIP2 to function and can be turned off by signaling pathways that deplete PIP2. This review discusses the dependence of ion channels on phosphoinositides and considers possible mechanisms by which PIP2 and analogues regulate ion channel activity.

Gene[97]	*Location*	*Function*
PIP3		
PI3K	3q26.3	Phosphatidylinositol 3-kinase is composed of an 85 kDa regulatory subunit and a 110 kDa catalytic subunit. The protein encoded by this gene represents the catalytic subunit, which uses ATP to phosphorylate PtdIns, PtdIns4P and PtdIns(4,5)P2. This gene has been found to be oncogenic and has been implicated in cervical cancers.
PTEN	10q23.3	This gene was identified as a tumor suppressor that is mutated in a large number of cancers at high frequency. The protein encoded this gene is a phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase. It contains a tensin like domain as well as a catalytic domain similar to that of the dual specificity protein tyrosine phosphatases. Unlike most of the protein tyrosine phosphatases, this protein preferentially dephosphorylates phosphoinositide substrates. It negatively regulates intracellular levels of phosphatidylinositol-3,4,5-trisphosphate in cells and functions as a tumor suppressor by negatively regulating AKT/PKB signaling pathway.
RAS	5q13.3	The protein encoded by this gene is located in the cytoplasm and is part of the GAP1 family of GTPase-activating proteins. The gene product stimulates the GTPase activity of normal RAS p21 but not its oncogenic counterpart. Acting as a suppressor of RAS function, the protein enhances the weak intrinsic GTPase activity of RAS proteins resulting in the inactive GDP-bound form of RAS, thereby allowing control of cellular proliferation and differentiation. Mutations leading to changes in the binding sites of either protein are associated with basal cell carcinomas. Mutations also have been associated with hereditary capillary malformations (CM) with or without arteriovenous malformations (AVM) and Parkes Weber syndrome. Alternative splicing results in two isoforms where the shorter isoform, lacking the N-terminal hydrophobic region but retaining the same activity, appears to be abundantly expressed in placental but not adult tissues.
RAF	3p25	This gene is the cellular homolog of viral raf gene (v-raf). The encoded protein is a MAP kinase kinase-kinase (MAP3K), which functions downstream of the Ras family of membrane associated GTPases to which it binds directly. Once activated, the cellular RAF1 protein can phosphorylate to activate the dual specificity protein kinases MEK1 and MEK2, which in turn phosphorylate to activate the serine/threonine specific protein kinases, ERK1 and ERK2. Activated ERKs are pleiotropic effectors of cell physiology and play an important role in the control of gene expression involved in the cell division cycle, apoptosis, cell differentiation and cell migration.
MAPK	22q11.21	The protein encoded by this gene is a member of the MAP kinase family. MAP kinases, also known as extracellular signal-regulated kinases (ERKs), act as an integration point for multiple biochemical signals, and are involved in a wide variety of cellular processes such as proliferation, differentiation, transcription regulation and development. The activation of this kinase requires its phosphorylation by upstream kinases. Upon activation, this kinase translocates to the nucleus of the stimulated cells, where it phosphorylates nuclear targets. Two alternatively spliced transcript variants encoding the same protein, but differing in the UTRs, have been reported for this gene
BMP	20p12	The protein encoded by this gene belongs to the transforming growth factor-beta (TGFB) superfamily. The encoded protein acts as a disulfide-linked homodimer and induces bone and cartilage formation

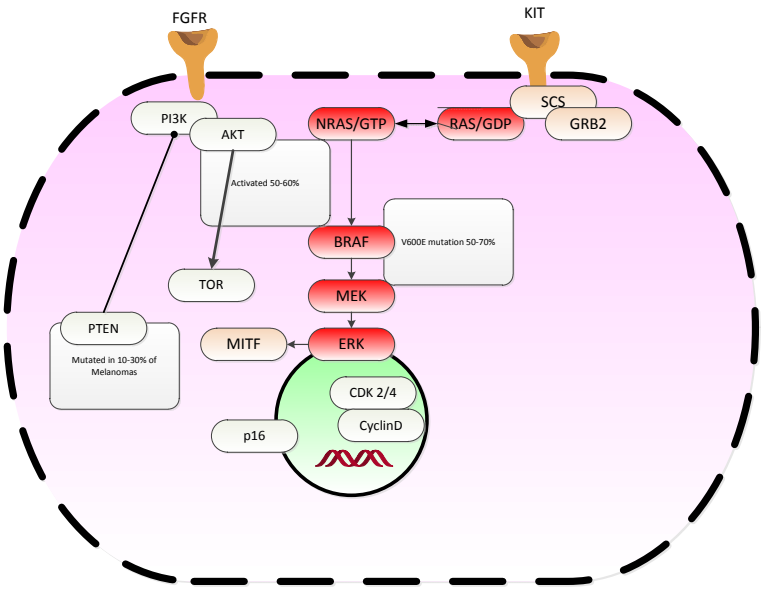
Gene[97]	*Location*	*Function*
β catenin	3p21	The protein encoded by this gene is part of a complex of proteins that constitute adherens junctions (AJs). AJs are necessary for the creation and maintenance of epithelial cell layers by regulating cell growth and adhesion between cells. The encoded protein also anchors the actin cytoskeleton and may be responsible for transmitting the contact inhibition signal that causes cells to stop dividing once the epithelial sheet is complete. Finally, this protein binds to the product of the APC gene, which is mutated in adenomatous polyposis of the colon. Mutations in this gene are a cause of colorectal cancer (CRC), pilomatrixoma (PTR), medulloblastoma (MDB), and ovarian cancer.
Wnt	7q31.2	This gene is a member of the WNT gene family. The WNT gene family consists of structurally related genes which encode secreted signaling proteins. These proteins have been implicated in oncogenesis and in several developmental processes, including regulation of cell fate and patterning during embryogenesis. Alternatively spliced transcript variants have been identified for this gene.
TCF	20q13.12	The protein encoded by this gene is a nuclear transcription factor which binds DNA as a homodimer. The encoded protein controls the expression of several genes, including hepatocyte nuclear factor 1 alpha, a transcription factor which regulates the expression of several hepatic genes. This gene may play a role in development of the liver, kidney, and intestines. Mutations in this gene have been associated with monogenic autosomal dominant non-insulin-dependent diabetes mellitus type I. Alternative splicing of this gene results in multiple transcript variants encoding several different isoforms.
TOR	1q21	The protein encoded by this gene is a DNA-binding transcription factor and is a member of the NR1 subfamily of nuclear hormone receptors. The specific functions of this protein are not known; however, studies of a similar gene in mice have shown that this gene may be essential for lymphoid organogenesis and may play an important regulatory role in thymopoiesis. In addition, studies in mice suggest that the protein encoded by this gene may inhibit the expression of Fas ligand and IL2. Two transcript variants encoding different isoforms have been found for this gene.
CREB	2q34	This gene encodes a transcription factor that is a member of the leucine zipper family of DNA binding proteins. This protein binds as a homodimer to the cAMP-responsive element, an octameric palindrome. The protein is phosphorylated by several protein kinases, and induces transcription of genes in response to hormonal stimulation of the cAMP pathway. Alternate splicing of this gene results in two transcript variants encoding different isoforms.
MITF	3p14.2	This gene encodes a transcription factor that contains both basic helix-loop-helix and leucine zipper structural features. It regulates the differentiation and development of melanocytes retinal pigment epithelium and is also responsible for pigment cell-specific transcription of the melanogenesis enzyme genes. Heterozygous mutations in this gene cause auditory-pigmentary syndromes, such as Waardenburg syndrome type 2 and Tietz syndrome. Alternatively spliced transcript variants encoding different isoforms have been identified.

Gene[97]	*Location*	*Function*
EGFR	7p12	The protein encoded by this gene is a transmembrane glycoprotein that is a member of the protein kinase superfamily. This protein is a receptor for members of the epidermal growth factor family. EGFR is a cell surface protein that binds to epidermal growth factor. Binding of the protein to a ligand induces receptor dimerization and tyrosine autophosphorylation and leads to cell proliferation. Mutations in this gene are associated with lung cancer. Multiple alternatively spliced transcript variants that encode different protein isoforms have been found for this gene.
IGF1R	15q26.3	This receptor binds insulin-like growth factor with a high affinity. It has tyrosine kinase activity. The insulin-like growth factor I receptor plays a critical role in transformation events. Cleavage of the precursor generates alpha and beta subunits. It is highly overexpressed in most malignant tissues where it functions as an anti-apoptotic agent by enhancing cell survival.
Her2	17q21.1	This gene encodes a member of the epidermal growth factor (EGF) receptor family of receptor tyrosine kinases. This protein has no ligand binding domain of its own and therefore cannot bind growth factors. However, it does bind tightly to other ligand-bound EGF receptor family members to form a heterodimer, stabilizing ligand binding and enhancing kinase-mediated activation of downstream signalling pathways, such as those involving mitogen-activated protein kinase and phosphatidylinositol-3 kinase. Allelic variations at amino acid positions 654 and 655 of isoform a (positions 624 and 625 of isoform b) have been reported, with the most common allele, Ile654/Ile655, shown here. Amplification and/or overexpression of this gene have been reported in numerous cancers, including breast and ovarian tumors. Alternative splicing results in several additional transcript variants, some encoding different isoforms and others that have not been fully characterized.

6 EXTRACELLULAR FACTORS AND MELANOMA: WNT, E-CADHERIN, THE ECM

Cell growth and cell movement are two characteristics of cancer. We examine this from two perspectives, one for melanoma and another for the benign condition called Dupuytren's disease which is a genetically related disease of excess growth of the fascia in the hands. Both are controlled by the Wnt gene product and both have a relationship with E cadherin which is a protein on the surface of the cell which causes adhesion of cells to cells. Also both Wnt and cadherin are extracellular. Namely Wnt flows between the cells and attaches to certain ligands and then if the intracellular elements are properly aligned the cell starts to proliferate. E cadherin is a surface protein which binds the cell to a certain location. When E cadherin fails then we see the cell start to move from where it is supposed to stay. Thus these two factors present a small picture on the loss of control which we observe in cancers.

The key issue is to understand pathways and points at which they break and where they can be controlled. From Murphy p 21 we have the following general example:



Although the above diagram deals not with Wnt or E Cadherin but the more classic sets of pathway elements we can see how progress can be made. PTEN for example is common in many cancers. We will discuss this at length later. It is seen somewhat in melanoma as well. BRAF is a major control point for melanoma and most of the recent work in pathway control has been focused here. It is possible to block this point which when the mutation occurs and it fails to control the cell reproduction we see uncontrolled growth. This type of model we will use over and over again.

Thus in the section we look briefly at two key characteristics of cancer, uncontrolled replication and uncontrolled movement, and examine two related mechanisms which control both actions. There will be many others we will introduce. These are useful as examples.

6.1 WNT

Wnt is a gene product which acts by adhering to surface proteins on the outside of a cell. By so adhering the Wnt can then influence the internal pathways in the cell often blocking apoptosis and initiating a cell growth and proliferation. In simple terms Wnt activates a pathway that allows β-catenin to enter the nucleus and activate a set of transcription promoters which in turn start the process of cell growth and proliferation.

The following is a typical Wnt pathway action:

1. Wnt is generated in a cell and is then secreted and in turn moves extra-cellularly to bind as a ligand on other cell surface receptors.

2. Wnt binds to the cell surface receptor Frizzled and it results in the activation of that receptor.

3. Activated Frizzled inhibits GSK-3 by means of the Disheveled protein. GSK-3 (Glycogen synthase kinase) normally inhibits β-catenin. This is a critical step because once activated β-catenin will result in a cascade of other actions resulting in cell growth. GSK normally activated phosphorylates β-catenin to keep it inactivated..

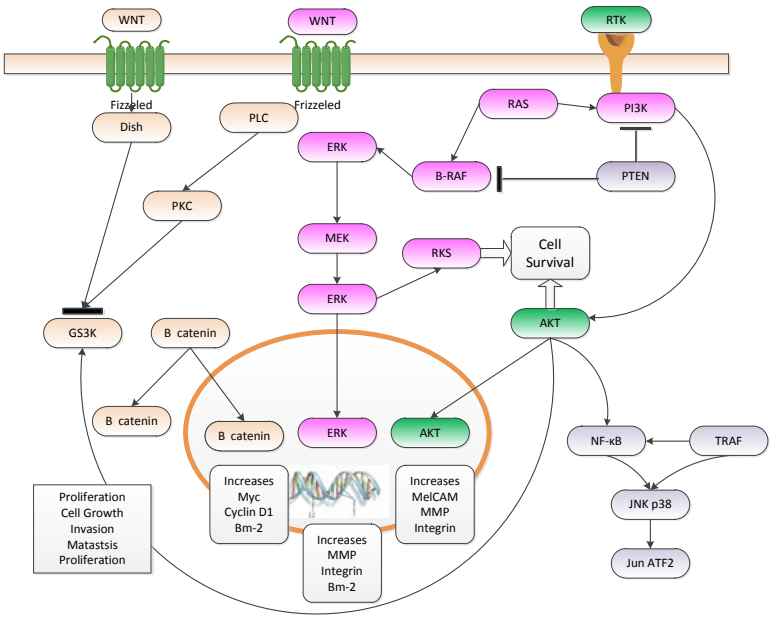
4. β-catenin now accumulates in the cytoplasm and at a certain concentration level β-catenin is transported into the nucleus.

5 When β-catenin is in the nucleus it activates TCF/LEF, a protein which is a transcription factor, and combined this results in the transcription of MYC, a strong proto-oncogene as well as CCDN1.

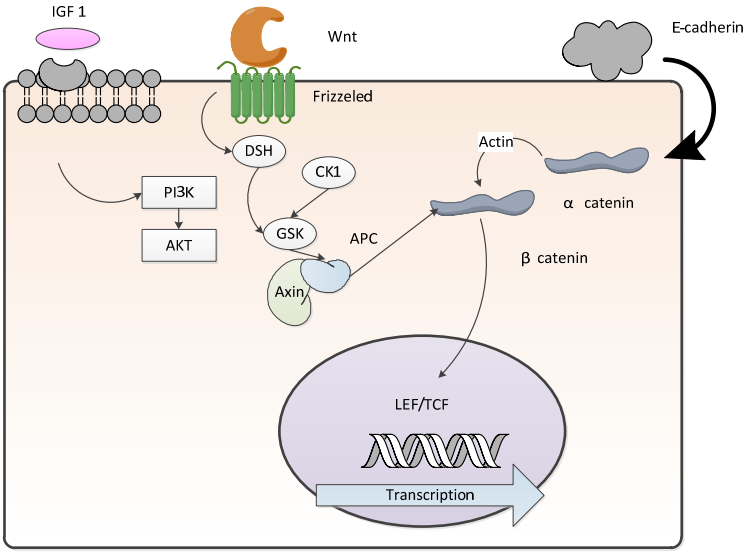
6. This results in uncontrolled cell growth and proliferation.

This is but one of many such pathways but one which is surface activated.

In the graphic below we depict some of the complex pathway processes and their effects. We show the catenin, ERK and Akt effects as each of their control mechanisms are affected. These are three of the major pathway challenges to normal cell homeostasis.



E cadherin is a set of molecules which are attached to the surface of cells and act in a manner to effect cell to cell adhesion. For example a melanocyte adheres to a keratinocyte in the basement membrane of the skin. If this adhesion fails for some reason then the melanocyte can start to wander off. When that happens and the melanocyte moves upward to the epidermis away from the basement layer we call that a melanoma in situ. The cells may not have yet gained the ability to reproduce in excess but they have lost a key element of a health melanocyte, namely the ability to stay fixed. In fact as we shall discuss the E cadherin is replaced by an N cadherin which often allows proliferating melanocytes to cluster together in groups and not have the simple keratinocyte structure.



As noted by Cavallaro and Christofori:

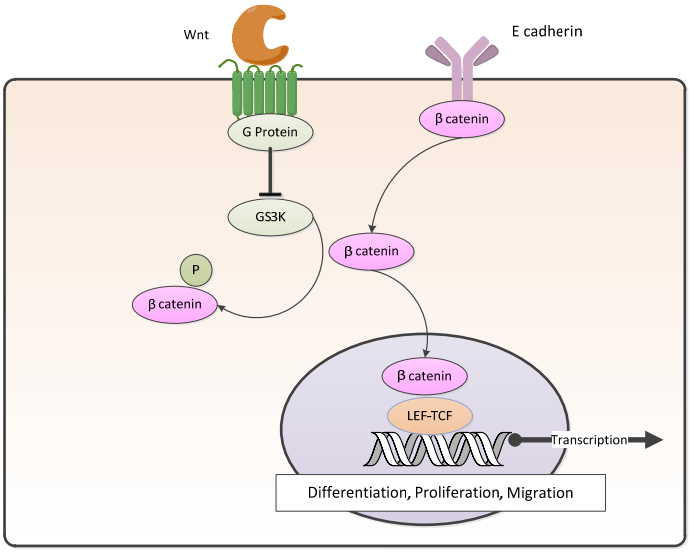
As well as their crucial role in assembling the E-cadherin- mediated cell-adhesion complex, β-catenin and γ-catenin also have important functions in the canonical WNT signalling pathway[Y]. Non-sequestered, free β-catenin and γ-catenin are rapidly phosphorylated by glycogen synthase kinase 3β (GSK-3β) in the adenomatous polyposis coli (APC)–axin–GSK-3β complex and are subsequently degraded by the ubiquitin–proteasome pathway. If the tumour suppressor APC is non-functional, as in many colon cancer cells, or if GSK-3β activity is blocked by the activated WNT-signalling pathway, β-catenin accumulates at high levels in the cytoplasm. Subsequently, it translocates to the nucleus, where it binds to members of the TCF/LEF1 family of transcription factors and modulates the expression of their target genes, including c-MYC, cyclin D1, fibronectin, MMP7, ID2, CD44, NrCAM, axin-2 (conductin), TCF1 and others, which are mostly genes implicated in cell proliferation and tumour progression. This dual function of β-catenin has motivated several experiments to address whether the loss of E-cadherin function would subsequently lead to the activation of the WNT signalling pathway.

We demonstrate some of this detail below. The catenin is attached to the E-cadherin which is released and if Wnt is activated then the GSK3 is blocked and it migrates to the nucleus where it induces cell proliferation.

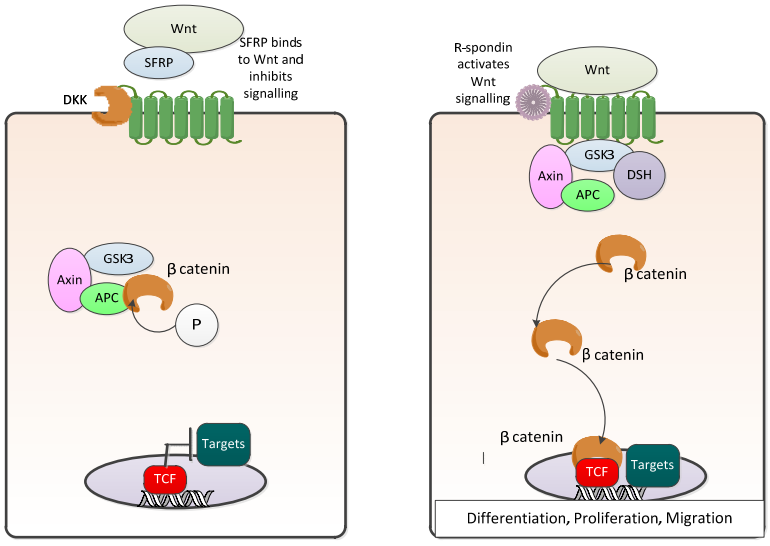
Now Wnt is a major component in many cells and especially those that are required for reproduction like bone marrow, colon cells, and skin cells. The control of the Wnt process is essential for homeostasis. Clearly one would want to have cells requiring proliferation to have a well regulated Wnt path. Thus keratinocytes need continual proliferation since the move from the basement membrane to the skin surface where they are sloughed away. However this would not be the case for melanocytes, where we need limited control. Melanocytes generally remain fixed at the basement membrane and movement or proliferation is inhibited.

The authors continue:

*In several human cancer types, including **melanoma, prostate and breast cancer**, loss of E-cadherin function is accompanied by de novo expression of mesenchymal cadherins, such as N-cadherin and cadherin-11 (OB-cadherin. Cadherin-11 is expressed in invasive breast cancer and in breast cancer cell lines, and a carboxy-terminally truncated, alternatively spliced form of cadherin-11 can induce an invasive phenotype even in E-cadherin-positive breast cancer cell line. Upregulated expression of P-cadherin in breast cancers and of cadherin-6 in renal cell carcinoma also correlates with poor prognosis. By contrast, T-cadherin (also known as H-cadherin) behaves more like E-cadherin: it is downregulated in basal and squamous-cell carcinomas of the skin, correlating with an invasive phenotyp. N-cadherin has been shown to promote cell motility and migration — an opposite effect to that of E-cadherin.*



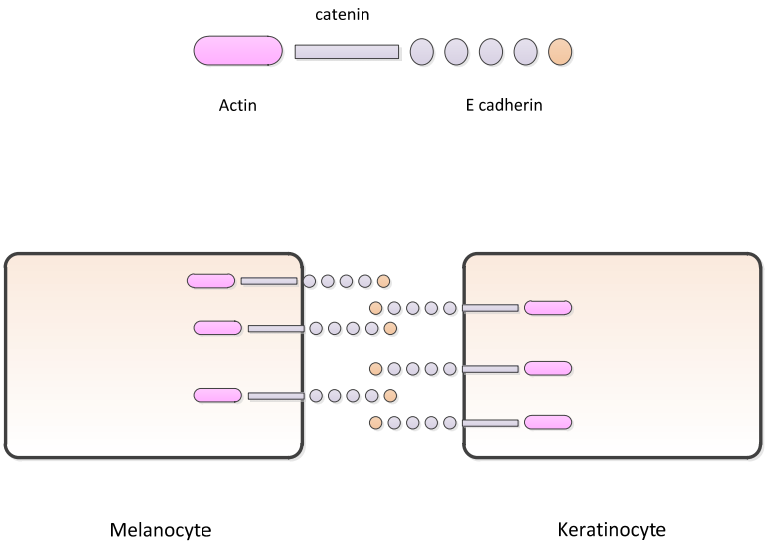
Adapted from Miller and Mihm NEJM July 2006



Modified from http://www.nejm.org/doi/full/10.1056/NEJMoa1101029#t=article

6.2 E CADHERIN

We now examine the cadherin structure. This we depict below as the bonding of two cells via the E cadherin elements which themselves are attached to a catenin and actin proteins within the cell wall. The E cadherin is a bonding protein which finds other specific bonding proteins and then attaches itself within a specified framework. Thus in the basement layer of the skin, at the bottom of the epidermis, the melanocyte attaches uniquely to a keratinocyte and fixes its position in the basement layer so as not to migrate.



E-cadherin is generated at 16q21.1. As is stated in MMMP[98]:

E-cadherin is one of the most important molecules of cell-cell adhesion in non-neural epithelial tissues. This 120 kDa transmembrane glycoprotein is generally localized on the surface of epithelial cells in a region of cell-cell contact that is known as the adherens junction. Cadherins are calcium-dependent cell adhesion proteins. They preferentially interact with themselves in a homophilic manner in connecting cells: cadherins may thus contribute to the sorting of heterogeneous cell types. CDH1 is involved in mechanisms regulating cell-cell adhesions, mobility and proliferation of epithelial cells. It is a ligand for integrin alpha-E/beta-7. Acts as a disulfide-linked homodimer. Interacts directly (via the cytoplasmic domain) with CTNNB1 or JUP to form the PSEN1/cadherin/catenin adhesion complex which connects to the actin skeleton through the actin binding of alpha-catenin. Interaction with PSEN1 cleaves CDH1 resulting in the disassociation of cadherin-based adherens junctions (CAJ). Anchored to actin microfilaments through association with alpha-catenin, beta-catenin and gamma-catenin. Sequential proteolysis induced by apoptosis or calcium influx, results in translocation from sites of cell-cell contact to the cytoplasm.

During apoptosis or with calcium influx, cleaved by a membrane-bound metalloproteinase (ADAM10), PS1/gamma-secretase and caspase-3 to produce fragments of about 38 kDa (E-CAD/CTF1), 33 kDa (E-CAD/CTF2) and 29 kDa (E-CAD/CTF3), respectively. Processing by the metalloproteinase, induced by calcium influx, causes disruption of cell-cell adhesion and the subsequent release of beta-catenin into the cytoplasm. The residual membrane-tethered cleavage product is rapidly degraded via an intracellular proteolytic pathway. Cleavage by caspase-3 releases the cytoplasmic tail resulting in disintegration of the actin microfilament system. The gamma-secretase-mediated cleavage promotes disassembly of adherens junctions.

In fact the β catenin is bound to the tail of the E cadherin complex and it is released when Wnt is activated. It is this release and movement to the nucleus which gives rise to proliferation.

[98] http://www.mmmp.org/MMMP/public/biocard/viewBiocard.mmmp?id=1301

6.3 WNT, E-CADHERIN AND DUPUTRYEN'S DISEASE

Duputryen's disease is a benign disorder of excess clustered nodular growth of the fibrin, and is a benign fibromatosis of the hand (see Dolmans et al and see Tubiana et al) . What seems to happen is the cells of fibroblasts are overly activated and bind together in clumps creating nodule like structures which then impinge on nerves and muscles impeding hand motion.

As Dolmans et al state:

The WNT gene family consists of structurally related genes that encode glycoproteins, extracellular signaling molecules. Abnormal Wnt signaling is linked to a range of diseases, especially cancer. The best-understood Wnt-signaling pathway is the canonical pathway, which activates the nuclear functions of β-catenin, leading to changes in gene expression that influence cell proliferation and survival.[18] Abnormal proliferation of fibroblasts is a key feature in the early development of Dupuytren's disease. The disease can be divided into three histologic stages:

stage 1, proliferation of fibroblasts;

stage 2, differentiation of fibroblasts into myofibroblasts; and

stage 3, formation of mature type 1 collagen.

Wnt signaling is known to regulate the proliferation and differentiation of fibroblasts in both cancer and fibromatosis. Most of our knowledge of Wnt signaling is derived from studies of cancer. In colon cancer, up-regulation of Wnt signaling causes intestinal crypt cells to proliferate for longer than usual before they migrate and differentiate. This prolonged proliferation phase results in the formation of polyps and confers a predisposition to cancer.

There is substantial evidence to show that this up regulation leading to proliferation is common in prostate cancer and melanoma as well.

They continue:

The Wnt proteins Wnt2, Wnt4, and Wnt7B, which were identified on GRAIL analysis, bind to frizzled receptors, leading to a cascade of events that eventually result in a decrease in the rate of β-catenin degradation. Secreted frizzled-related proteins, such as SFRP4, antagonize the Wnt-signaling pathway by binding to either Wnts or frizzled receptors, thereby affecting receptor occupancy. In the absence of active Wnt, β-catenin is degraded, and potential target genes will not be activated.

We depict the signalling below. Note in the inhibited case we have an extracellular SFRP binding to Wnt and preventing it from activating the pathway as a ligand. Also not that what is driving this is Wnt 2,4 and 7B. Melanoma id driven by Wnt 5.

6.4 COMMONALITY OR COINCIDENCE

6.5 BRAF AND WNT

Recently we have seen targeted drugs to control BRAF.

In the paper by Biechele et al we have:

Because the Wnt/β-catenin signaling pathway is linked to melanoma pathogenesis and to patient survival, we conducted a kinome small interfering RNA (siRNA) screen in melanoma cells to expand our understanding of the kinases that regulate this pathway. We found that BRAF signaling, which is constitutively activated in many melanomas by the $BRAF^{V600E}$ mutation, inhibits Wnt/β-catenin signaling in human melanoma cells. Because inhibitors of $BRAF^{V600E}$ show promise in ongoing clinical trials, we investigated whether altering Wnt/β-catenin signaling might enhance the efficacy of the $BRAF^{V600E}$ inhibitor PLX4720. We found that endogenous β-catenin was required for PLX4720-induced apoptosis of melanoma cells and that activation of Wnt/β-catenin signaling synergized with PLX4720 to decrease tumor growth in vivo and to increase apoptosis in vitro. This synergistic enhancement of apoptosis correlated with reduced abundance of an endogenous negative regulator of β-catenin, AXIN1. In support of the hypothesis that AXIN1 is a mediator rather than a marker of apoptosis, siRNA directed against AXIN1 rendered resistant melanoma cell lines susceptible to apoptosis in response to treatment with a $BRAF^{V600E}$ inhibitor. ***Thus, Wnt/β-catenin signaling and AXIN1 may regulate the efficacy of inhibitors of $BRAF^{V600E}$, suggesting that manipulation of the Wnt/β-catenin pathway could be combined with BRAF inhibitors to treat melanoma.***

We now know that if a person has the BRAF V600E presence that use of xxx will manage the melanoma for a period.

6.6 THE EXTRA CELLULAR MATRIX

The focus on intracellular pathways has been a prime direction of research in the development of cancers. However there has from time to time been some focus on the extracellular matrix, the "ECM", which relates in many ways to the stability of the cell, its localization. Cancer cells lose this sense of localization and begin to move.

The processes at play in the ECM have a significant impact on the processes that occur within a cell. Thus it is essential to have an understanding of the ECM. Recent work by Fisher and his people on MDA-9, a controller of certain ECM elements, demonstrates a control path that influences the internal pathways. We discuss the ECM in the context of the MDA-9 developments.

In this section we use a recent development in understanding the impact of Mda-9 and the nexus with the extra cellular matrix, ECM, and the control of metastatic melanoma.

We first review the Fisher Team efforts as recently presented and then we examine the standard intracellular pathways that have been examined and from that we provide an overview of the extra cellular matrix, ECM, which is the "glue" binding together cells and facilitating cell to cell communications.

We find this an interesting focus or research for several reasons:

1. It examines the ECM which has received limited focus.

2. It focuses on pathways as we have been also doing and specifically an interesting adjunct to the current B-RAF approach.

3. It establishes a clear path forward which is logically and experimentally based and verifiable.

There has been limited prior research on these issues. In Hearing and Leong, 380-386, there is a limited discussion regarding the ECM and melanoma with references. The work by Zent and Pozzi provides a broad and detailed perspective of the ECM with many cancers. However their work is not specific to melanoma. In Weinberg there are references but there does not appear to be any singular focus on the ECM as a standalone system element.

6.6.1 Recent Evidence

In a recent paper by Das et al, the authors (from Fisher's Lab at Virginia Commonwealth) state[99]:

Melanoma differentiation associated gene-9 (MDA-9), also known as syntenin, functions as a positive regulator of melanoma progression and metastasis. In contrast, the Raf kinase inhibitor RKIP, a negative modulator of RAF-stimulated MEKK activation, is strongly downregulated in metastatic melanoma cells. In this study, we explored an hypothesized inverse relationship between MDA-9 and RKIP in melanoma. Tumor array and cell line analyses confirmed an inverse relationship between expression of MDA-9 and RKIP during melanoma progression.

We found that MDA-9 transcriptionally downregulated RKIP in support of a suggested crosstalk between these two proteins. Further, MDA-9 and RKIP physically interacted in a manner that correlated with a suppression of FAK and c-Src phosphorylation, crucial steps necessary for MDA-9 to promote FAK/c-Src complex formation and initiate signaling cascades that drive the MDA-9-mediated metastatic phenotype.

Lastly, ectopic RKIP expression in melanoma cells overrode MDA-9-mediated signaling, inhibiting cell invasion, anchorage-independent growth and in vivo dissemination of tumor cells.

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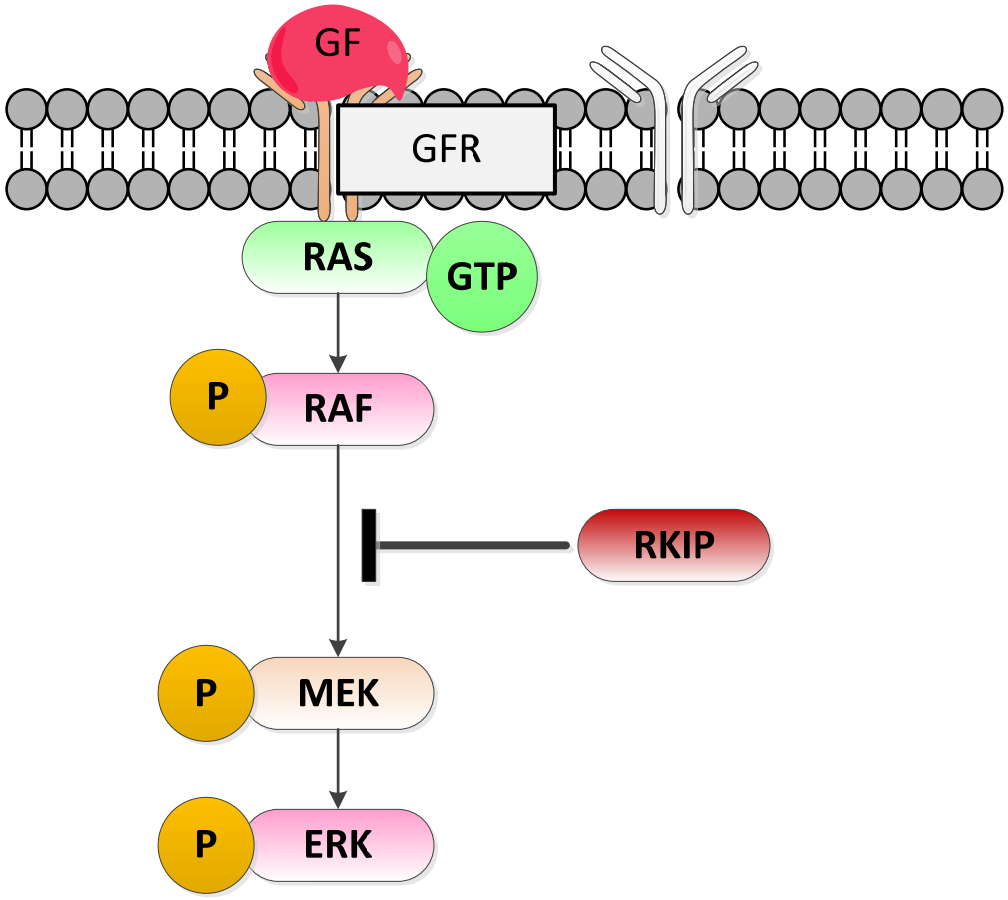
99 http://cancerres.aacrjournals.org/search?author1=Swadesh+K+Das&sortspec=date&submit=Submit ; Therapeutics, Targets, and Chemical Biology Raf Kinase Inhibitor RKIP Inhibits MDA-9/Syntenin-Mediated Metastasis in Melanoma, Das, S., et al, *Cancer Res Published Online First October 11, 2012.*

Taken together, these findings establish RKIP as an inhibitor of MDA-9-dependent melanoma metastasis, with potential implications for targeting this process therapeutically.

From the paper by Houben et al we have the RKIP activation as shown below:



As Houben et al state:

The Ras/Raf/MEK/ERK intracellular signalling cascade is a major determinant in the control of cell growth, differentiation, and survival and can be activated in response to a variety of extracellular stimuli. Stimulation of growth factor receptors results in the activation of the small G-protein Ras, which in turn interacts with the protein kinase Raf leading to its activation. MAP kinase kinase kinase (Raf) phosphorylates and activates MAP kinase kinase (MEK), and MEK phosphorylates and activates extracellular signal-regulated kinase (ERK) 1/2 (p42/p44 MAP kinases).

Although Raf and MEK appear largely restricted to only one class of substrates, ERK targets more than 70 substrates including membrane, cytoskeletal, cytoplasmic, nuclear, and even mitochondrial proteins. Recently, a negative regulator of this pathway has been described. The Raf Kinase Inhibitor Protein (RKIP) binds to either Raf or MEK and thereby interferes with the activation of MEK by Raf. The importance of the Ras/Raf/MEK/ERK signalling pathway for

carcinogenesis is well established. Indeed, Ras genes (K-ras, H-ras, and N-ras) are the most frequently mutated oncogenes detected in human cancer.

Houben et al further state about RKIP (12q24.23) as a target the following:

To assess the relevance of the Ras/Raf/MEK/MAP kinase pathway, we analyzed for activating B-Raf mutations and we elucidated the presence of the Raf Kinase Inhibitor Protein (RKIP) and extracellular signal-regulated kinase (ERK) as well as the phosphorylation status of ERK. All MCC samples were negative for the B-Raf$_{V600E}$ mutation. Remarkably, RKIP, which was shown to interfere with the activation of MEK by Raf, was highly expressed in primary as well as in metastatic MCC. … Western blot analysis of three MCC-derived cell lines revealed in one case the pattern present in situ (i.e. high RKIP expression and complete absence of phosphorylated ERK).

Thus the Fisher team seems to seek out a RKIP inhibitor to slow the pathway. This is in addition to the B-RAF inhibitors which are currently in clinical use.

Now in an industry piece on the same article the author Ho states[100]:

…. the scientist believes that they have the ability to eliminate melanoma differentiation associated gene-9 (mda-9)/syntenin, a specific protein. In the experiment, the researchers discovered that Raf kinase inhibitor protein (RKIP) was able to interact and suppress with mda-9/syntenin. The protein was originally cloned in a laboratory and past studies showed how it interacted with c-Src, another protein, to produce a set of chemical reactions that later boosted metastasis.

"Prior research suggests that RKIP plays a seminal role in inhibiting cancer metastasis, but, until now, the mechanisms underlying this activity were not clear," explained Paul Fisher, the program co-leader of Cancer Molecular Genetics at Virginia Commonwealth University Massey Cancer Center, in a prepared statement. "In addition to providing a new target for future therapies, there is potential for using these two genes as biomarkers for monitoring melanoma development and progression."

The team of investigators discovered that RKIP become attached to mda-9/syntenin, which resulted in limiting the expression of mda-9/syntenin. With the finding of this physical interaction, the scientists believe that they could possibly create small molecules that are similar to RKIP and the molecules could be used as drugs to treated metastasis in cancers like melanoma.

We depict this pathway below:

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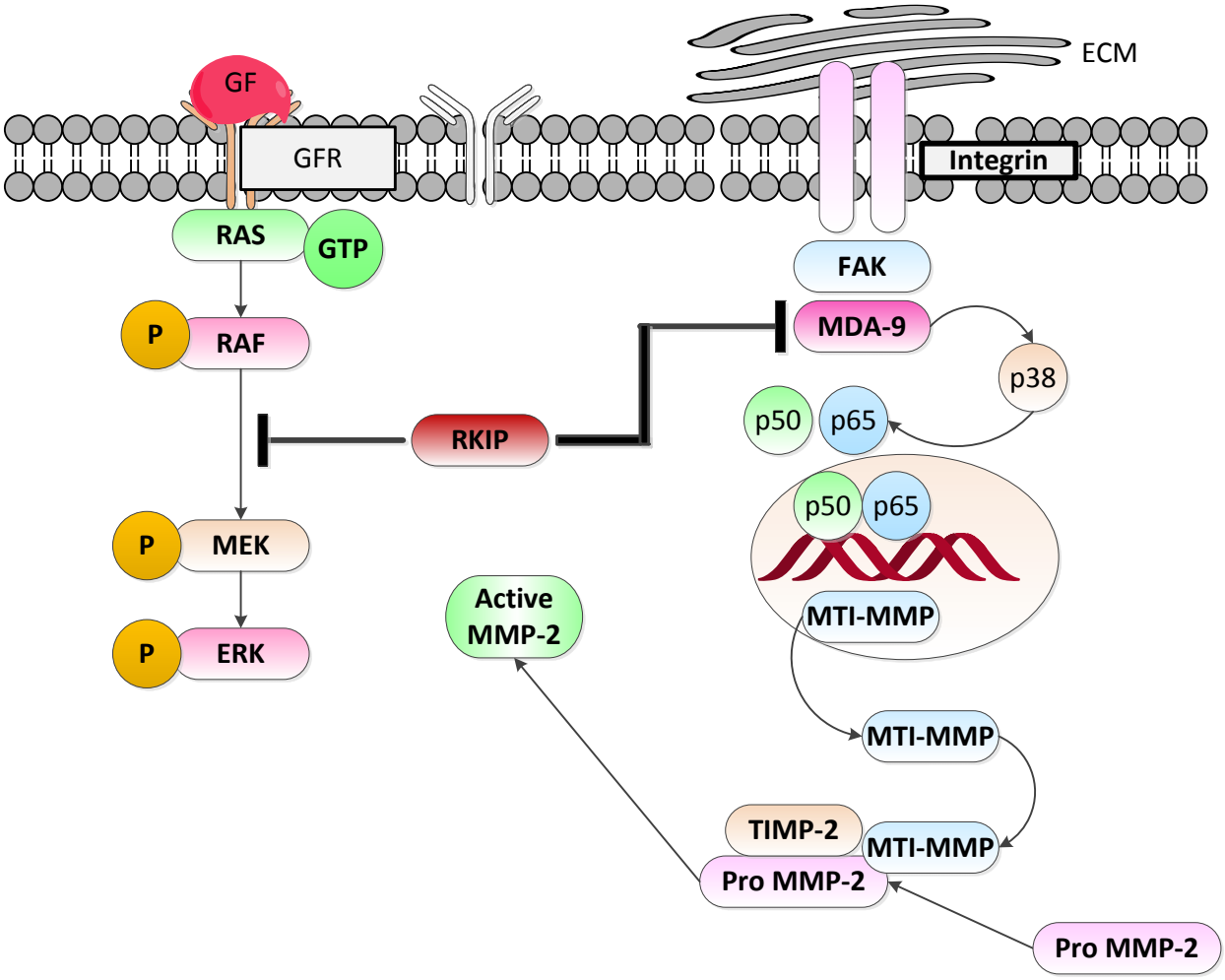
[100] http://www.redorbit.com/news/health/1112732493/stopping-the-spread-of-melanoma-by-removing-protein-affecting-metastasis/ ; Ho, C., Stopping The Spread Of Melanoma By Removing Protein Affecting Metastasis, RedOrbit, November 15, 2012



The article continues:

There was also a difference in terms of the level of mda-9/syntenin and RKIP. While malignant and metastasis melanoma cells had higher levels of mda-9/sytnenin compared to RKIP, the healthy melanocyte cells that create pigment in eyes, hair, and skin had higher levels of RKIP than mda-9/syntenin. The researchers believe that different levels in the proteins could be used in diagnosis, particularly in following the progression of a disease or tracking a patient's response to a particular treatment.

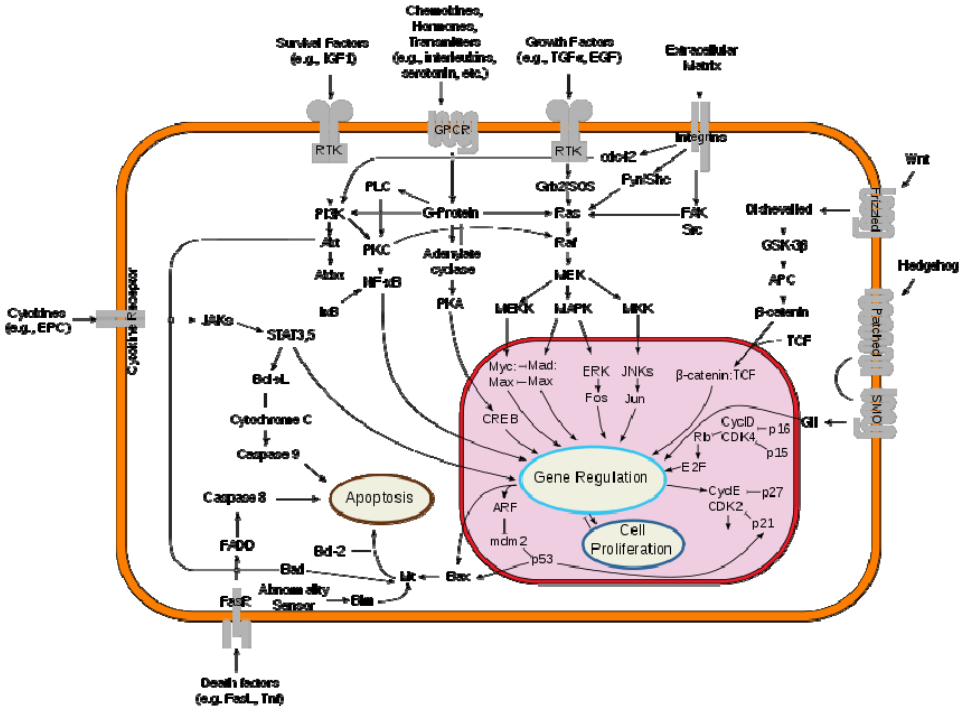
"Our findings represent a major breakthrough in understanding the genetic mechanisms that lead to metastasis in melanoma. Prior studies have shown that levels of mda-9/syntenin are elevated in a majority of cancers, including melanoma, suggesting that our findings could be applicable for a wide range of diseases," continued Fisher, who also serves as chairman of VCU's Department of Human and Molecular Genetics and director of the VCU Institutes of Molecular Medicine, in the statement.

Moving forward, the scientists plan to determine how they can develop small molecules that mimic RKIP. These molecules could potentially be utilized in new treatments for melanoma.

This is a fundamental result. It demonstrates another pathway element and at the same time connects the intracellular pathways with the extra cellular matrix and their pathways. Potentially this is diagnostic, prognostic and a treatment as well.

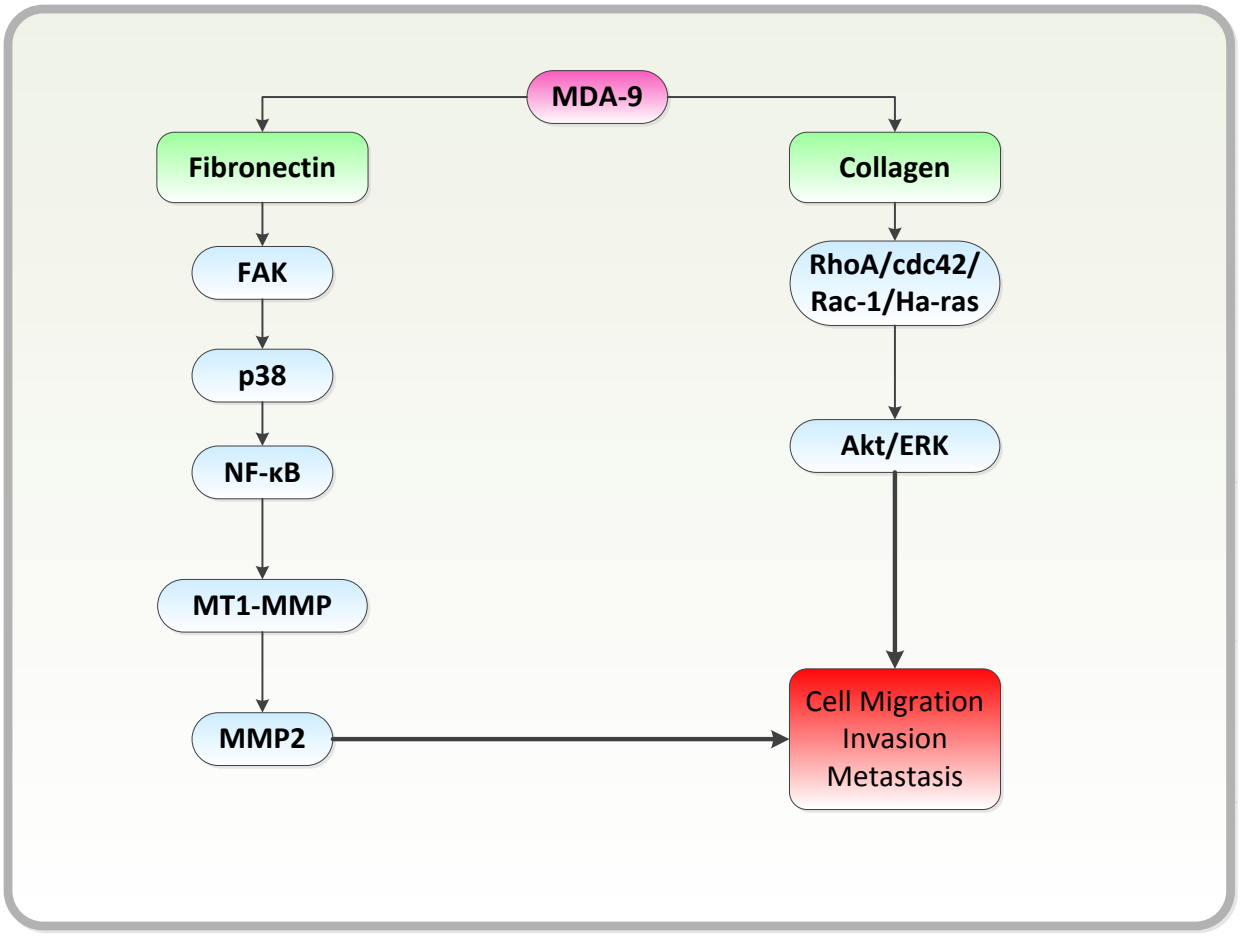
6.6.2 Standard Intra-Cellular Pathways

The following Figure is a repetition of the standard intra-cellular pathways. We have discussed these at length.



What is different from what we have detailed previously is the Extra Cellular Matrix connection via the integrins. This yields the controlling FAK path using FAK and Src. Note that this activates RTK and Ras and thus as we have described many of the other internal pathways this is the first time we have involved the ECM directly. The ECM is a significant element in cancer proliferation, it is the sea in which the changing cells sail metaphorically but at the same time it allows communication with the environment as well as presenting ligands to receptors.

As depicted in Sarkar et al, we have the following sets of paths and the results:



We shall be examining these in some detail. Let us first characterize some of the above identified elements controlled by the extracellular matrix path. The others we have examined in detail elsewhere.

6.6.3 FAK

FAK is also known as; PTK2, FADK; FAK1; FRNK; PPP1R71; p125FAK; pp125FAK. It is located at 8q24.3. It is a kinase.

NCBI states its function as follows:

This gene encodes a cytoplasmic protein tyrosine kinase which is found concentrated in the focal adhesions that form between cells growing in the presence of extracellular matrix constituents. The encoded protein is a member of the FAK subfamily of protein tyrosine kinases but lacks significant sequence similarity to kinases from other subfamilies. Activation of this gene may be an important early step in cell growth and intracellular signal transduction pathways triggered in response to certain neural peptides or to cell interactions with the extracellular matrix. Several transcript variants encoding different isoforms have been found for this gene, but the full-length natures of only three of them have been determined.

6.6.4 Src

SRC is located at 20q12-q13. As noted in NCBI[101]:

This gene is highly similar to the v-src gene of Rous sarcoma virus. This proto-oncogene may play a role in the regulation of embryonic development and cell growth. The protein encoded by this gene is a tyrosine-protein kinase whose activity can be inhibited by phosphorylation by c-SRC kinase. Mutations in this gene could be involved in the malignant progression of colon cancer. Two transcript variants encoding the same protein have been found for this gene.

6.6.5 p38

The p38 gene has multiple names. It is MAPK14, RK; CSBP; EXIP; Mxi2; CSBP1; CSBP2; CSPB1; PRKM14; PRKM15; SAPK2A; p38ALPHA. It is located at 6p21.3-p21.2.

Its function described by NCBI is as follows[102]:

The protein encoded by this gene is a member of the MAP kinase family. MAP kinases act as an integration point for multiple biochemical signals, and are involved in a wide variety of cellular processes such as proliferation, differentiation, transcription regulation and development.

This kinase is activated by various environmental stresses and proinflammatory cytokines.

The activation requires its phosphorylation by MAP kinase kinases (MKKs), or its autophosphorylation triggered by the interaction of MAP3K7IP1/TAB1 protein with this kinase. The substrates of this kinase include transcription regulator ATF2, MEF2C, and MAX, cell cycle regulator CDC25B, and tumor suppressor p53, which suggest the roles of this kinase in stress related transcription and cell cycle regulation, as well as in genotoxic stress response.

Four alternatively spliced transcript variants of this gene encoding distinct isoforms have been reported.

6.6.6 NF-κB

We have discussed this before. We reiterate what that discussion contains.

NF-κB is a transcription factor that resides in the cytoplasm. It is called Nuclear Factor and was identified by David Baltimore as an enhancer factor for the κ chain of Ig light chain in B lymphocytes. When activated it moves to the nucleus and is a transcription factor in activating over 400 genes. It is activated by a large number of stimuli and its action of a large gene set causes significant DNA activity. NF-κB appears on 10q24 and is somatic and acts in a dominant manner.

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[101] http://www.ncbi.nlm.nih.gov/gene/6714

[102] http://www.ncbi.nlm.nih.gov/gene/1432

In a recent paper by Zhang et al they state:

The majority of tumors progressing during androgen deprivation therapy (referred to here as androgen deprivation- resistant prostate cancer or ADRPC) express higher levels of AR transcript and protein suggesting that a marked increase in AR expression is a critical event in therapy resistance…

Recent studies also demonstrate that increased AR expression is both necessary and sufficient to convert prostate cancer growth from a hormone therapy-sensitive to a resistant state in xenograft models... Since AR mRNA levels are often increased in ADRPC without gene amplification , …

it is likely mediated by transcription factors and transcription regulating signal transduction pathways that are altered during progression.

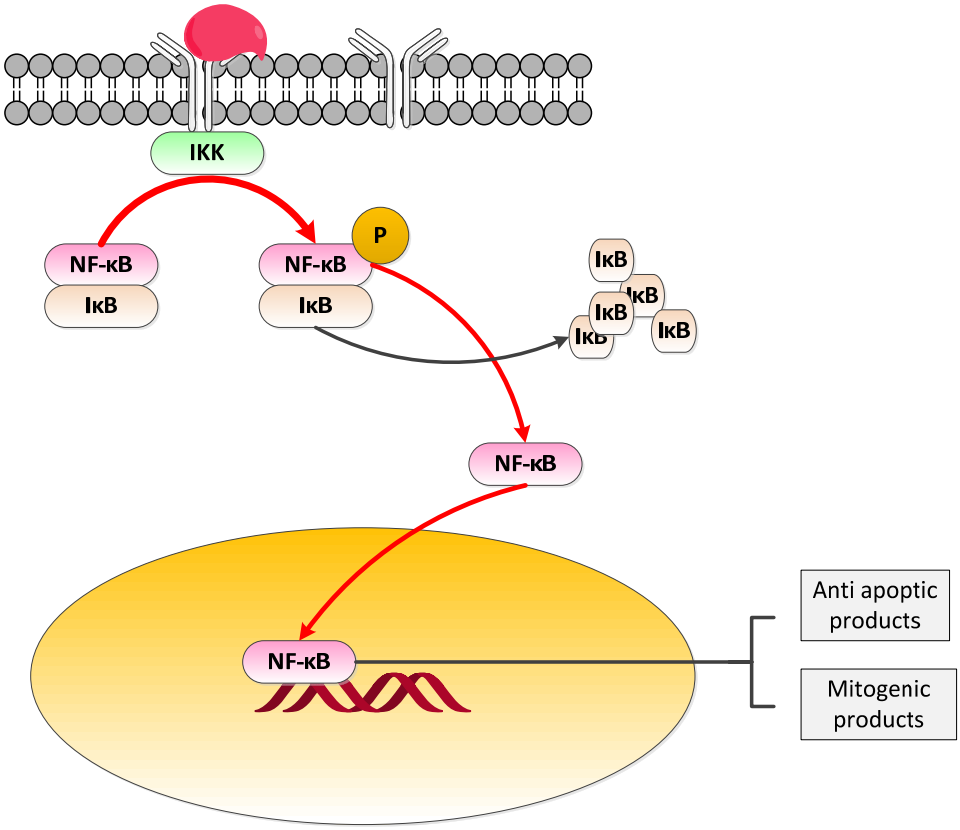
Nuclear Factor (NF)-κB is a family of transcription factors composed of homo- and hetero-dimers initially identified as an enhancer binding protein for the immunoglobulin light chain in B lymphocytes…

Zhang continues:

Several studies have examined the expression of NF-κB in human prostate cancer and its relationship to clinical features of the disease. NF-κB/p65 is overexpressed in prostatic intraepithelial neoplasia and cancer compared with benign epithelium. Nuclear levels of NF-κB/p65 correlate with NF-κB-dependent expression of BclII, cyclin D1, matrix metalloproteinase-9, and vascular endothelial growth factor.

Recent work indicates that NF-κB/p65 expression is predictive of biochemical recurrence in patients with positive surgical margins after radical prostatectomy and nuclear localization of NF-κB is increased in prostate cancer lymph node metastasis and can be used to predict patient outcome. These results demonstrate that NF-κB/p65 is frequently activated in human prostate adenocarcinoma and expression may be related to progression.

We now depict this putative pathway based upon the work of Kwang and Aggarwal. This is shown below. Activated NF-κB is clearly an activator of an anti-apoptosis process in the nucleus. The paper by Huang et al shows that blockade of NF-κB is an effective suppressor of angiogenesis, invasion and metastasis of prostate cancer.



NF-κB is another transcription protein seen in melanoma. This protein is characterized by:

- NF-κB is a transcription factor that resides in the cytoplasm.
- It is called Nuclear Factor and was identified by David Baltimore as an enhancer factor for the κ chain of Ig light chain in B lymphocytes
- When activated it moves to the nucleus and is a transcription factor in activating over 400 genes
- It is activated by a large number of stimuli and its action of a large gene set causes significant DNA activity
- NF-κB appears on 10q24 and is somatic and acts in a dominant manner.

NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) is a protein complex that controls the transcription of DNA. NF-κB is widely used by eukaryotic cells as a regulator of genes that control cell proliferation and cell survival. As such, many different types of human tumors have mis-regulated NF-κB: that is, NF-κB is constitutively active. Active NF-κB turns on the expression of genes that keep the cell proliferating and protect the cell from conditions that would otherwise cause it to die via apoptosis.

As Amiri and Richmond state:

Nuclear Factor-kappa B (NF-κB) is an inducible transcription factor that regulates the expression of many genes involved in the immune response. Recently, NF-κB activity has been shown to be upregulated in many cancers, including melanoma. Data indicate that the enhanced

activation of NF- κB may be due to deregulations in upstream signaling pathways such as Ras/Raf, PI3K/Akt, and NIK. Multiple studies have shown that NF-κB is involved in the regulation of apoptosis, angiogenesis, and tumor cell invasion, all of which indicate the important role of NF-κB in tumorigenesis. Thus, understanding the molecular mechanism of melanoma progression will aid in designing new therapeutic approaches for melanoma.

They continue:

Constitutive activation of NF-κB is an emerging hallmark of various types of tumors including breast, colon, pancreatic, ovarian, and melanoma [9–14]. In the healthy human, NF-κB regulates the expression of genes involved in normal immunologic reactions (e.g. generation of immunoregulatory molecules such as antibody light chains) in response to proinflammatory cytokines and by-products of microbial and viral infections [15–17]. NF-κB also modulates the expression of factors responsible for growth as well as apoptosis. However, increased activation of NF-κB results in enhanced expression of proinflammatory mediators, leading to acute inflammatory injury to lungs and other organs, and development of multiple organ dysfunctions as well as cancer.

They then summarize NF-kB's role in melanoma as:

3.1. Apoptosis resistance and cell proliferation*: In processes such as tumor initiation and promotion where prolonged survival of cells is a crucial event, NF-κB plays an important role as a mediator of inhibition of apoptosis. In melanoma, NF-κB has been shown to activate expression of anti-apoptotic proteins such as tumor necrosis factor receptor-associated factor 1 (TRAF1), TRAF2, and the inhibitor-of apoptosis (IAP) proteins c-IAP1, c-IAP2, and melanoma inhibitor of apoptosis (ML-IAP), survivin as well as Bcl-2 like proteins…*

3.2. Invasion and metastasis*: In invasion and metastasis of melanoma, NF-κB may regulate the production of prostaglandins via cyclooxygenase-2 (COX-2), which has been shown to be overexpressed in melanoma [44,45]. It was shown that COX-2 is expressed in the majority of primary malignant melanoma, as well as in five human malignant melanoma cell lines….*

However as Liu et al (2006) state:

Malignant melanoma is the most lethal skin cancer, whose ability to rapidly metastasize often prevents surgical cure.

Furthermore, the systemic treatment of melanoma is largely ineffective due to the intrinsic resistance of melanoma cells to numerous anticancer agents. Increased survival of melanoma cells is primarily attributed to the constitutive activation of the transcription factor nuclear factor kB (NF-kB), which regulates the expression of many anti-apoptotic, pro-proliferative and pro-metastatic genes.

Canonical activation of the NF-kB pathway occurs when NF-kB switches its localization from the cytoplasm, where it is maintained inactive by assembly with the inhibitor IkB protein, to the nucleus, where NF-kB regulates gene expression. NF-kB activation relies upon the

phosphorylation dependent ubiquitination and degradation of IkB mediated by the IkB kinase (IKK) complex and b-Trcp E3 ubiquitin ligases.

Consequently, both IKK activity and the levels of b-Trcp regulate the extent of IkB degradation and hence NF-kB activation. The genetic basis that underlies the elevated NF-kB activity in malignant melanoma largely remains elusive.

Constitutively active IKK has been demonstrated to sustain NF-kB activation in human melanoma cells, resulting in induction of the chemokine CXCL1. CXCL1, in turn, is capable of activating IKK and NF-kB and promoting cell survival and tumorigenesis …. However, the original genetic alterations that initiate this feed-forward mechanism in melanoma remain unclear.

One of the major oncogenic events described in the genesis of malignant melanoma is constitutive activation of the Ras-regulated RAF-MEK-ERK mitogen-activated protein kinase (MAPK) pathway. This is achieved most frequently by activating mutations in either BRAF (e.g. V600E substitution) or, less frequently, in N-RAS … Recent evidence indicates that oncogenic BRAF activity is essential for human melanoma cell growth and survival …

However, despite prior reports that RAF can activate NF-kB …, the mechanism(s) by which $BRAF_{V600E}$ ($BRAF_{VE}$) may elicit NF-kB signaling in melanoma cells have not yet been elucidated. Activation of the canonical NF-kB pathway depends on both IKK activity, which has been shown to be elevated in human melanomas….

Liu et al conclusion is speculative but telling:

Taken together, these data support a model in which mutational activation of BRAF in human melanomas contributes to constitutive induction of NF-κB activity and to increased survival of melanoma cells.

Again we have the issue of speculation as to where and why the mutations occur. Here they speculate about the BRAF mutation resulting in the antiapoptotic control with NF-κB.

6.6.7 MMP-9

As NCBI states[103]:

Proteins of the matrix metalloproteinase (MMP) family are involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling, as well as in disease processes, such as arthritis and metastasis. Most MMP's are secreted as inactive proproteins which are activated when cleaved by extracellular proteinases.

[103] http://www.ncbi.nlm.nih.gov/gene/4318

The enzyme encoded by this gene degrades type IV and V collagens. Studies in rhesus monkeys suggest that the enzyme is involved in IL-8-induced mobilization of hematopoietic progenitor cells from bone marrow, and murine studies suggest a role in tumor-associated tissue remodeling

We shall discuss MMP in detail when we summarize the ECM.

6.6.8 cdc42

As NCBI states[104]:

The protein encoded by this gene is a small GTPase of the Rho-subfamily, which regulates signaling pathways that control diverse cellular functions including cell morphology, migration, endocytosis and cell cycle progression. This protein is highly similar to Saccharomyces cerevisiae Cdc 42, and is able to complement the yeast cdc42-1 mutant.

The product of oncogene Dbl was reported to specifically catalyze the dissociation of GDP from this protein. This protein could regulate actin polymerization through its direct binding to Neural Wiskott-Aldrich syndrome protein (N-WASP), which subsequently activates Arp2/3 complex. Alternative splicing of this gene results in multiple transcript variants.

6.7 THE EXTRACELLULAR MATRIX

The ECM has often been neglected when discussing cancer pathways. Weinberg has multiple references but does not seem to place it in any specific spotlight. In Lewin, Cell, the discussion is quite well focused but yet there is but passing reference to the impact on cancer pathways. Specifically in Lewin on p 850 there is reference to MMP-9, here a metalloproteinase, and melanoma[105].

The ECM is the collection of molecules that lie between the cell walls. The ECM provides for structural integrity as well as facilitates and even participates in cell to cell communications. The ECM is a highly complex and quite active element in the ongoing life of the cells. In addition we all too often look to what happens in a cell, with at best a nod to ligands, and we do not look at the cell internals as well as the ECM as a holistic system totality. The work of the Fisher Team in a small way may help refocus this effort on the complex as a working whole.

We will follow Lewin and deal with the principal participants in the ECM. There are a wealth of books which focus on this area.

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104 http://www.ncbi.nlm.nih.gov/gene/998

105 As NCBI states: "*Proteins of the matrix metalloproteinase (MMP) family are involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling, as well as in disease processes, such as arthritis and metastasis. Most MMP's are secreted as inactive proproteins which are activated when cleaved by extracellular proteinases. The enzyme encoded by this gene degrades type IV and V collagens. Studies in rhesus monkeys suggest that the enzyme is involved in IL-8-induced mobilization of hematopoietic progenitor cells from bone marrow, and murine studies suggest a role in tumor-associated tissue remodeling.*" see http://www.ncbi.nlm.nih.gov/gene/4318

6.7.1 Collagen

Collagens provide structure support. They are triple helical proteins wrapped to provide that supporting structure between the cells. There any many types of collagen and the actually assembly commences within the cell and the semi-finished product passes through the cell wall to the ECM. For our purposes the collagen complexes are at this time of limited interest.

6.7.2 Fibronectin

Fibronectin facilitates the process of connecting cells to matrices of collagen. Fibronectin proteins have a six element structure. Cells bind to fibronectin via receptors called integrins. The fibronectin binding thus activates pathways within the cell, thereby establishing an intra and intercellular pathway complex. The pathways activated control growth, movement and cell differentiation.

We can now examine some of the relevant literature on fibronectin and melanomas. As Yi and Ruoslahti state:

Fibronectin is a prototypic extracellular matrix (ECM) protein that is deposited by various types of cells into an adhesive fibrillar meshwork of protein (1). Fibronectin, and ECM in general, control many cellular functions, including growth, migration, differentiation, and survival. The signals that control these behaviors are transmitted from the ECM to the cell by integrins, a family of transmembrane receptors (2, 3). Malignant cells often bypass the ECM–integrin signaling system; they are not bound by the spatial constraints imposed by the ECM on normal cells, and they no longer require ECM contact for survival

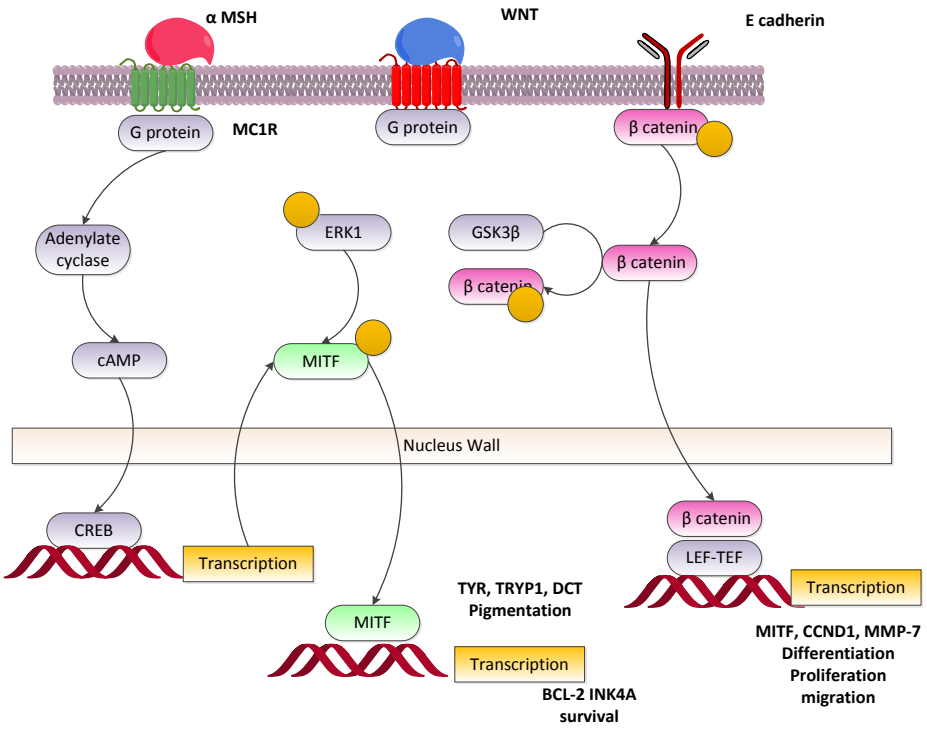
Liu et al state:

Tumor cells frequently exhibit decreased adhesiveness due to failure to deposit stromal fibronectin (FN), permitting more rapid proliferation, migration, invasion, and metastasis. Although up-regulation of FN has been noted in gene profiles of carcinomas compared with normal tissue, reduced FN expression has been described at the peripheral margins of invading tumors. In this study, we investigate the role of FN in cancer behavior. … Neoplastic transformation is often characterized by changes in the organization of the cytoskeleton, decreased cell adhesion, and aberrant adhesion–mediated signaling (2). Disruption of normal cell adhesion contributes to enhanced proliferation, migration, and invasion leading to metastasis. Fibronectin (FN) is an extracellular matrix protein with putative roles in mediating these actions. Indeed, tumor cells with decreased adhesiveness frequently fail to deposit stromal FN (3). In particular, reduced FN expression has been noted in transformed cell lines and primary tumors (4), including thyroid cancer (3, 5, 6), where diminished FN has been identified at the periphery of invasive tumor margins. In this context, we found that down-regulation of FN stimulates thyroid cancer cell proliferation and tumor growth (7). Conversely, 1, 25-dihydroxy vitamin D3 treatment increases cell adhesiveness and inhibits cell proliferation and tumor growth through enhanced FN expression.

We will come back to fibronectin in out later analysis.

6.7.3 E-cadherin

We have discussed E-cadherin at length in previous work. It plays a critical role in stabilizing cell adhesion and localization. Loss of E-cadherin results in loss of cell localization and thus cell movement. Specifically in melanocytes the cells begin to leave the basal layer and migrate upward as in melanoma in situ and downward as in superficial spreading melanoma.



As Swiatoniowski et al state:

Integrins are molecules which play a significant role in cell-extracellular matrix (ECM) interactions. They interact with the RGD tripeptide of fibronectin (FN), one of the main components of ECM. Labile expression of FN has been proven to play an important role both in the normal developmental process (morphogenetic movements) and in the course of carcinogenesis … Many authors have implicated loss or decrease of EC expression as an independent negative prognostic marker in breast cancer patients (6-9). There is increasing experimental evidence for a relationship between the EC level and different features of breast cancer, including histological grade (7, 16) and axillary lymph node involvement (13-16)…. In conclusion, our experiment revealed no prognostic value for EC or FN expressions in a homogenous group of patients

6.7.4 Proteoglycan

Proteoglycans are single polypeptide with multiple sugars attached. They provide for hydration in the ECM.

6.7.5 Protease

The proteases are ECM proteins which function to degrade the refuse in the ECM. The metalloproteinases are a family of proteases. They are also called MMP. MMP-9 and MMP-2 are ones of the MMPs often associated with melanoma.

There has been extensive work examining the MMPs and melanoma some dating back to the 1990s, see that of Luca et al. A recent result by Hoffman et al state:

Matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) are involved in tumour progression and metastasis. In this study, we investigated the in vitro and in vivo expression patterns of MMP-1, MMP-2, MMP-3, MMP-9, TIMP-1 and TIMP-2 mRNA and protein in a previously described human melanoma xenograft model. This model consists of eight human melanoma cell lines with different metastatic behaviour after subcutaneous (s.c.) injection into nude mice. MMP-1 mRNA was detectable in all cell lines by reverse transcription polymerase chain reaction (RT-PCR), but the expression was too low to be detected by Northern blot analysis. No MMP-1 protein could be found using Western blotting. MMP-2 mRNA and protein were present in all cell lines, with the highest expression of both latent and active MMP-2 in the highest metastatic cell lines MV3 and BLM. MMP-3 mRNA was expressed in MV3 and BLM, and in the non-metastatic cell line 530, whereas MMP-3 protein was detectable only in MV3 and BLM.

None of the melanoma cell lines expressed MMP-9. TIMP-1 and TIMP-2 mRNA and protein, finally, were present in all cell lines. A correlation between TIMP expression level and metastatic capacity of cell lines, however, was lacking. MMP and TIMP mRNA and protein expression levels were also studied in s.c. xenograft lesions derived from a selection of these cell lines. RT-PCR analysis revealed that MMP-1 mRNA was present in MV3 and BLM xenografts, and to a lesser extent in 530. Positive staining for MMP-1 protein was found in xenograft lesions derived from both low and high metastatic cell lines, indicating an in vivo up-regulation of MMP-1. MMP-2 mRNA was detectable only in xenografts derived from the highly metastatic cell lines 1F6m, MV3 and BLM. In agreement with the in vitro results, the highest levels of both latent and activated MMP-2 protein were observed in MV3 and BLM xenografts.

With the exception of MMP-9 mRNA expression in 530 xenografts, MMP-3, MMP-9, and TIMP-1 mRNA and protein were not detectable in any xenograft, indicating a down-regulated expression of MMP-3 and TIMP-1 in vivo. TIMP-2 mRNA and protein were present in all xenografts; interestingly, the strongest immunoreactivity of tumour cells was found at the border of necrotic areas. Our study demonstrates that of all tested components of the matrix metalloproteinase system, only expression of activated MMP-2 correlates with increased malignancy in our melanoma xenograft model, corroborating an important role of MMP-2 in human melanoma invasion and metastasis.

We shall see the impact of MMPs as we examine the pathways.

6.7.6 Integrins

Integrins are for the most part the receptors for ECM proteins. They are one of many such cell surface receptors. The integrins play important roles in cell homeostasis and cell to cell communications.

6.7.7 MDA-9

Let us briefly examine the gene MDA-9 and its protein Mda-9 and what is known and how it has evolved. Now MDA-9 is located on (8q12). As the NIH data base states:

The protein encoded by this gene was initially identified as a molecule linking syndecan-mediated signaling to the cytoskeleton. The syntenin protein contains tandemly repeated PDZ domains that bind the cytoplasmic, C-terminal domains of a variety of transmembrane proteins. This protein may also affect cytoskeletal-membrane organization, cell adhesion, protein trafficking, and the activation of transcription factors.

The protein is primarily localized to membrane-associated adherens junctions and focal adhesions but is also found at the endoplasmic reticulum and nucleus. Alternative splicing results in multiple transcript variants encoding different isoforms[106].

In the paper, Src kinase activation is mandatory for MDA-9/syntenin-mediated activation of nuclear factor-κB, by H Boukerche, H Aissaoui, C Prévost, H Hirbec, S K Das, Z-Z Su, D Sarkar and P B Fisher, the author's state:

The scaffolding postsynaptic density-95/disks large/zonula occludens-1 (PDZ) domain-containing protein melanoma differentiation associated gene-9 (MDA-9)/syntenin is a tandem PDZ protein overexpressed in human melanoma, and breast and gastric cancer cells. MDA-9/syntenin affects cancer cell motility and invasion through distinct biochemical and signaling pathways, including focal adhesion kinase and p38 mitogen-activated protein kinase (MAPK), resulting in activation of the nuclear factor (NF)-κB pathway.

MDA-9/syntenin also promotes melanoma metastasis by activating c-Src, but how c-Src regulates NF-κB activation is unclear. Using a human melanoma model, we document that MDA-9/syntenin–c-Src interactions are positive regulators of NF-κB activation. Inhibition of c-Src by PP2 treatment, by blocking c-Src or mda-9/syntenin expression with small interfering RNA, or in c-Src (−/−) knockout cell lines, reduces NF-κB activation following overexpression of mda-9/syntenin or c-Src.

Deletion or point mutations of the PDZ binding motif preventing MDA-9/syntenin association with c-Src reveals that both PDZ domains, with PDZ2 being the dominant module, are required for activating downstream signaling pathways, including p38 MAPK and NF-κB. We also document that MDA-9/syntenin–c-Src complexes functionally cooperate with NF-κB to promote

[106] http://www.ncbi.nlm.nih.gov/gene/6386

anchorage-independent growth, motility and invasion of melanoma cells. These findings underscore PDZ domains of MDA-9/syntenin as promising potential therapeutic targets for intervening in a decisive component of cancer progression, namely, metastatic tumor spread[107]*….*

(MDA-9 Acts as a PDZ domain-containing adapter protein. In adherens junctions, it couples syndecans to cytoskeletal proteins or signaling components. Seems to be required for the targeting of TGF-alpha to the cell surface in the secretory pathway. By virtue of its association with a large number of additional proteins, including class B ephrins, TGF-alpha, phosphotyrosine phosphatase, neurofaschin, neurexin, schwannomin/merlin, IL-5 receptor, various glutamate receptor subtypes, and the syndecan family of heparan sulfate proteoglycans, MDA9 has been implicated in diverse processes, including protein trafficking, activation of the transcription factor SOX4, cytoskeleton-membrane organization, and cell adhesion/migration….

(MDA-9) Its expression is induced by IFN-gamma in melanoma cells. Is believed to be involved in cancer metastasis. In melanoma, it promotes the metastatic phenotype by activating NFkB and focal adhesion kinase (FAK), which promotes induction of matrix metalloproteinase (MMP) and then migration and extracellular matrix invasion of melanoma cells. Syntenin is overexpressed and promotes cell migration in metastatic human breast and gastric cancer cell lines.

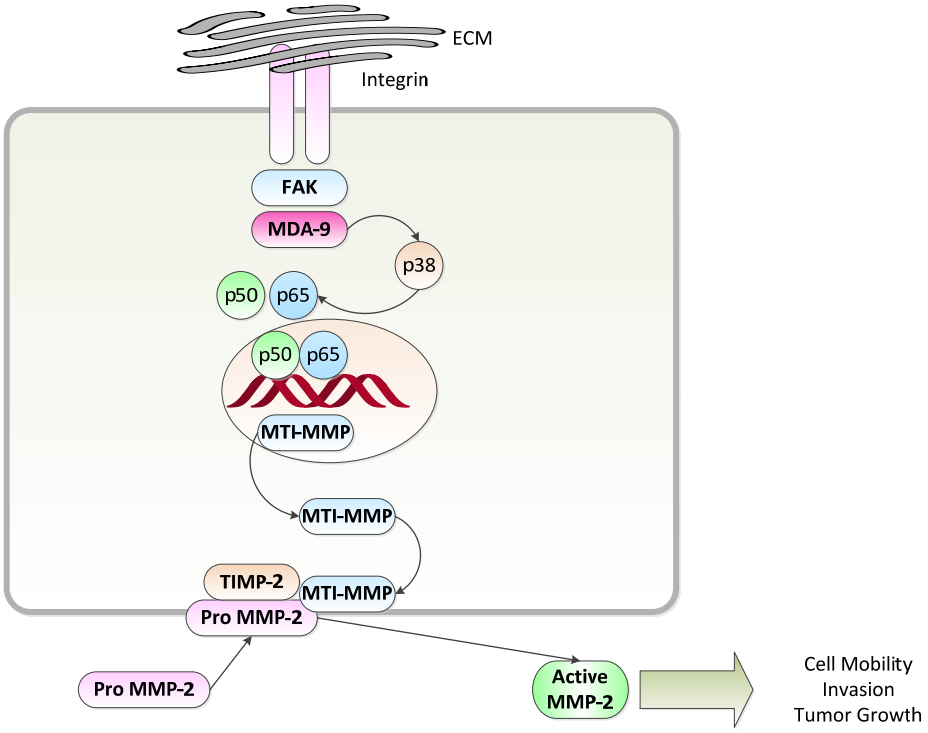
The gene product is also called by many other names, specifically:

1. MDA9
2. MDA-9
3. TGF alpha cytoplasmic domain interacting protein18
4. TACIP18
5. SYCL
6. Syntenin-1
7. Syndecan binding protein 1
8. SDCBP
9. Melanoma differentiation associated protein 9

From Das et al. we have the following modified figure[108]:

[107] http://www.nature.com/onc/journal/v29/n21/pdf/onc201065a.pdf

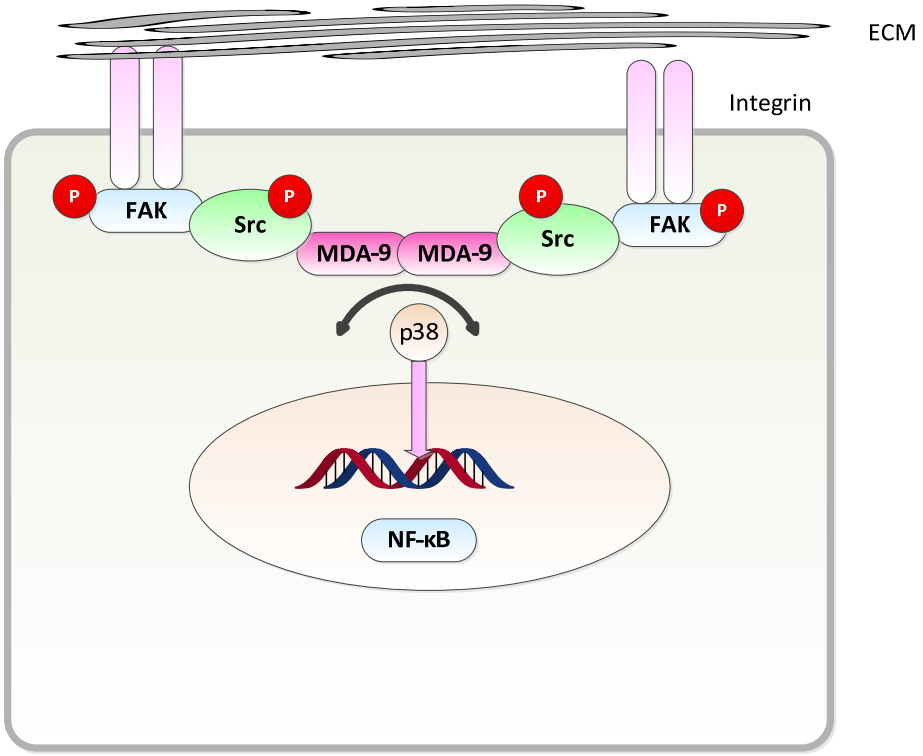
[108] http://www.bioscience.org/2012/v17/af/3911/fulltext.asp?bframe=figures.htm&doi=yes



Das et al state regarding the above pathway model:

Schematic diagram for mda-9/syntenin mediated NFκB activation. Upon interaction with ECM (fibronectin), MDA-9/syntenin activates the p38/MAPK by augmenting FAK phosphorylation. This results in degradation of IκBα and movement of p65 from the cytoplasm where interaction with p50 results in binding to target genes (MT1-MMP) resulting in enhanced production of MT1-MMP, which interacts with TIMP-2 activating pro-MMP-2 to produce active MMP-2. This product then enhances cell motility, invasion, and cancer cell growth. mda-9/Syntenin activates the NF-kB pathway.

The original Figure appears to be from Boukerche et al as shown with some mods below:



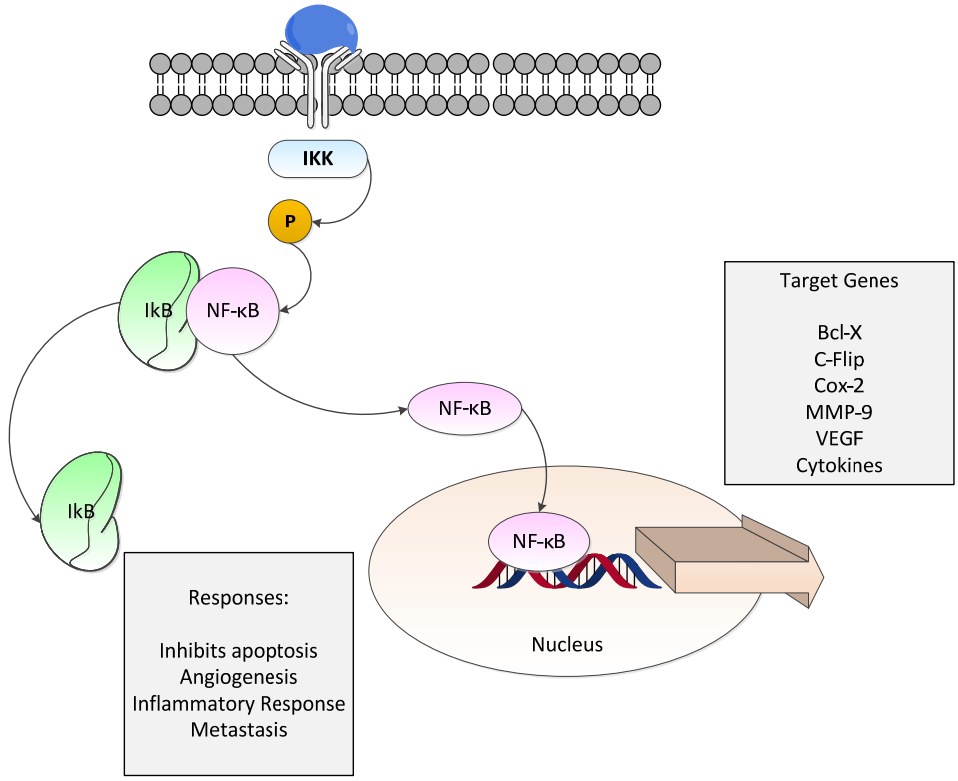
Note the differences. First the original shows multiple integrins and multiple FAK binding and in turn a binding of MDA-9 initiating the p38 pathway. Also note the explicit presence of NF-κB and its result of genes forcing mobility, invasion and metastasis. The authors state:

Hypothetical model of signal transduction pathways coordinately regulated by MDA-9/syntenin through its interaction with c-Src. MDA-9/ syntenin interaction with c-Src results in clustering of c-Src/FAK signaling complexes at high concentrations on the plasma membrane. The activated c-Src/FAK complexes activate the p38 MAPK/NF-κB pathways that regulate expression of genes involved in migration and invasion and thus play a crucial role in MDA-9/syntenin-mediated tumor progression.

The initiation of NF-κB is a significant factor since this transcription factor is what appears to be the instigator of the metastatic processes.

From Pecorino, p 220, we have again presented the details (as modified)[109]:

[109] Pecorino, Molecular Biology of Cancer, Oxford (New York) 2nd Ed, 2005.



The above graphic clearly demonstrates the movement of the transcription factor into the nucleus, from a bound state with IkB to an unbound and active state. The target genes indicated includes an MMP gene which again goes into the ECM.

As Sarkar et al state:

*Melanoma differentiation associated gene-9 (*mda-*9), also known as syntenin, is a PDZ domain–containing adapter protein that is involved in organization of protein complexes in the plasma membranes, regulation of B-cell development, intracellular trafficking and cell-surface targeting, synaptic transmission, and axonal outgrowth. Recent studies now define a seminal role for* mda-*9/sytenin in cancer metastasis*.

Thus, Sarkar who is part of Fisher's Lab at Virginia, have had a focus on Mda-9. They continue:

Adapter proteins play an essential role in modulating signal transduction from the extracellular environment to the intracellular milieu by virtue of their association with key regulatory molecules … mda-*9 was originally cloned as a gene differentially expressed in human melanoma cells reprogrammed to terminally differentiate by combination treatment with IFN-*h *and the protein kinase C activator mezerein … Analysis of the subcellular distribution of* mda-*9/syntenin revealed its localization at the areas of cell-cell contact in cells of epithelial origin in colocalization with F-actin, syndecan-1, E-cadherin,* h*-catenin, and* a*-catenin (12). In fibroblasts,* mda-*9/ syntenin localizes to focal adhesions and in stress fibers. Overexpression of* mda-*9/syntenin in different cells induces the formation of plasma membrane structures,*

including ruffles, lamellipodia, fine extensions, and neurite-like structures, showing its role in regulating the structure and function of the plasma membrane…

They continue:

The major characteristic of malignant tumor cells is their ability to invade foreign tissues and form metastatic foci at distant locations in the body. Such a process requires tumor cell attachment to various matrix proteins, degradation of the extracellular matrix (ECM) mainly by matrix metalloproteinases (MMP), followed by migration into the surrounding stroma by tumor cells…A model of progression of melanoma suggests that it begins by conversion of a normal melanocyte into a benign nevi, subsequent transformation into a radial and then a vertical growth phase primary melanoma, and finally evolution into a metastatic melanoma.

Finally Sarkar et al outline the overall set of functions which MDA-9 is involved in. Specifically they state:

1. ***Interleukin-5 signaling.*** *mda-9/syntenin interacts with interleukin- 5 (IL-5) receptor* a *and the transcription factor Sox4, thus mediating IL-5–induced Sox4 activation …*

2. ***Cell-surface trafficking.*** *Although* mda-*9/syntenin is located predominantly in the plasma membrane, it is also identified in the early secretory pathway such as the endoplasmic reticulum, intermediate compartment, and* cis-*Golgi, thus facilitating cellsurface trafficking of secreted molecules such as proTGF-*a*, an epidermal growth factor receptor ligand…*

3. ***mda-9/syntenin and ephrin signaling.*** *Ephrins and their cellsurface tyrosine kinase receptors are implicated in controlling axon guidance and fasciculation …*

4. ***Mediation of cohesiveness of epidermal stem cells.*** *In the basal layer of interfollicular epidermis the stem cells are clustered, a feature known as cohesiveness. These cells express high levels of Notch ligand D1, which is important for maintaining cohesiveness …*

5. ***Regulation of glutamate signaling.*** *The excitatory neurotransmitter glutamate interacts with its cognate receptors and regulates postsynaptic excitatory currents. Glutamate receptors interact with* mda-*9/syntenin, …*

6. ***Regulation of axon outgrowth.*** *Unc51.1 is a serine/threonine kinase that is important for neurite extension/parallel fiber formation in cerebellar granule neurons.* mda-*9/syntenin interacts with Unc51.1 and Rab5, a member of the Ras-like small GTPases that is a marker of early endosomes and is essential for endocytic membrane fusion and trafficking. …*

Boukerche et al in 2005 stated:

Studies using an enhanced green fluorescent protein mda-9*/ syntenin fusion protein showed that endogenous* mda-9*/syntenin colocalized with the E-cadherin complex and syndecan-1 at adherens junctions as well as with focal adhesions and stress fibers at cell-substratum contact in*

fibroblastic and epithelial cells. These findings suggest that Mda-9*/syntenin might promote cytoskeletal organizational changes and intracellular signaling.*

The organization of these dissimilar focal contacts is complex but was shown not only to contain the appropriate integrin but also cytoskeletal proteins (vinculin, talin, and a*-actinin) as well as several cytoplasmic protein tyrosine kinases, including members of the src family and focal adhesion kinase (FAK). Despite extensive research documenting an ability of* mda-9*/syntenin to form multivalent interactions, little is known about the role of* Mda-9*/syntenin in cancer development.*

Boukerche et al (2008) state:

Prior studies confirm that Mda-9*/syntenin stimulates motility through pathways involving FAK, p38MAPK, and NF-κB, leading to secretion of MMP-2 (4, 9). However, despite these intriguing observations, it is not fully understood how* Mda-9*/syntenin orchestrates these signaling molecules to enhance cancer cell motility and metastasis. A complex network of protein-protein interactions characterizes the structural organization of focal adhesions, involving known signaling molecules that play functional roles in various cellular activities and other less well-defined pathways.*

We presently show that Mda-9*/syntenin interacts with c-Src through its PDZ domain and activates the c-Src/FAK signaling pathway to maximize tumor cell motility and anchorage-independent growth of melanoma cells.* Mda-9*/Syntenin levels directly correlate with increased c-Src activity in a human melanoma model that closely mimics the early events of metastasis in humans.*

In 2010 Boukerche et al report (also in Fisher's Lab):

MDA-9/syntenin affects cancer cell motility and invasion through distinct biochemical and signaling pathways, including focal adhesion kinase and p38 mitogen-activated protein kinase (MAPK), resulting in activation of the nuclear factor (NF)-kappaB pathway.

MDA-9/syntenin also promotes melanoma metastasis by activating c-Src, but how c-Src regulates NF-kappaB activation is unclear. Using a human melanoma model, we document that MDA-9/syntenin-c-Src interactions are positive regulators of NF-kappaB activation. Inhibition of c-Src by PP2 treatment, by blocking c-Src or mda-9/syntenin expression with small interfering RNA, or in c-Src (-/-) knockout cell lines, reduces NF-kappaB activation following overexpression of mda-9/syntenin or c-Src.

Deletion or point mutations of the PDZ binding motif preventing MDA-9/syntenin association with c-Src reveals that both PDZ domains, with PDZ2 being the dominant module, are required for activating downstream signaling pathways, including p38 MAPK and NF-kappaB. We also document that MDA-9/syntenin-c-Src complexes functionally cooperate with NF-kappaB to promote anchorage-independent growth, motility and invasion of melanoma cells.

These findings underscore PDZ domains of MDA-9/syntenin as promising potential therapeutic targets for intervening in a decisive component of cancer progression, namely, metastatic tumor spread.

6.8 OBSERVATIONS

This set of papers from the Fisher Lab present several interesting connections between the ECM and the intra-cellular signaling paths. We have had prior arguments that one can develop models for metastasis by examining the cell as a target entity and then by modeling the environment, both the ECM and surrounding cells as influences on the target cell. In this work we can expand it to include ECM factors in some detail.

The suggested control of other pathway elements, beyond just the B-RAF control that we now have may be proven productive. Notwithstanding it does establish a research path that is based upon established cell dynamics.

7 MELANOMA AND CANCER STEM CELLS

The cancer stem cell theory has been developed over the past decade or so. For many years the theory was that cancer was clonal, namely one single cell was at fault and its progeny were the direct result of that genetically modified parent, a single parent, and that as the cancer evolved there may be increased genetic defects but again all were from a single parent.

Cancer stems cells are a construct which predicates the development of mature cells in a cell line as coming from a set of stem cells, akin to the blood cells arising from the bone. In contrast to the linear model of Vogelstein, say in the colon, the epithelial cell of the colon wall has some genetic disruption, and after multiple disruptions this epithelial cell becomes cancerous, dividing without bounds and failing to remain where is was supposed to. Typically an adenoma develops which after the final genetic hit becomes an adenocarcinoma.

For example, we have examined the prostate cancer cell, and in so doing have used a non CSC model, namely it is a basal or luminal cell which becomes genetically changed. If however we are wrong and there is an equivalent prostate cancer stem cell, as some have conjectured, then management of cancer of the prostate is quite a different thing. As we have expressed before, if one has diffuse HGPIN in the prostate and then after several high density prostate biopsies it disappears, is that inferentially valid for a prostate CSC?

The cancer stem cell construct is fundamentally different. It is not a mature cell which takes the genetic hits but the stem cell. The malignant stem cell acts almost as a force at a distance, and can impact other cells as the stem cell itself can reproduce, albeit at a somewhat slower rate than what it may influence.

Arguably if one can remove the stem cell then one removes any future malignancy, even to the extent of having other cells enter apoptosis for failure of having an active stem cell.

As Weinberg notes, there is the theory of clonal development of cancer which states that the cancer cells are pluripotent and have developed from a single source and that they have the capability of reproducing and do so in an autonomous manner[110]. Then there is the theory of the cancer stem cell, the theory which states that there is the equivalent of a stem cell as we know in blood cells, which have the capability but that the majority of malignant cells do not necessarily have that capacity.

The NCI presents an excellent summary of Cancer stem cell, CSC, research[111]:

The theory of the cancer stem cell (CSC) has generated as much excitement and optimism as perhaps any area of cancer research over the last decade. Biologically, the theory goes, these cells are distinct from the other cells that form the bulk of a tumor in that they can self-perpetuate and produce progenitor cells, the way that traditional stem cells do. The progenitors'

[110] Weinberg, Cancer, pp 416-417.

[111] http://www.cancer.gov/ncicancerbulletin/072710/page4

job is then to repopulate tumor cells eradicated by treatments such as chemotherapy or radiation.

But for all the attention and fanfare CSC research has received, the findings reported to date are far from clear-cut, investigators acknowledge. For example, most of the studies that have identified human CSCs have used mouse xenograft assays and cells from only a small number of human tumor samples, making it difficult to draw firm conclusions. In addition, other researchers haven't always been able to replicate initially reported findings. And while these tumor-initiating cells, as they are also called, have been described as being a rare class, several studies have found that the number of cells that can form tumors in these mouse experiments is actually quite large, suggesting that perhaps CSCs aren't such a privileged breed.

As we shall discuss herein, the CSC does not yet have a steady state definition or description. Furthermore it is also difficult to tag and identify. We shall discuss some of these issues. Now the NCI goes on to say:

In other words, the idea of just what cancer stem cells are, and their role in different cancers, appears to be changing.

"The [stem cell] model has not been adequately tested in most cancers," said Dr. Sean Morrison, who directs the Center for Stem Cell Biology at the University of Michigan. "I think that there are some cancers that do clearly follow a cancer stem cell model…But it will be more complicated than what's been presented so far."

They continue by noting a significant conclusion of the CSC theory, the fact that the CSC is the controlling cell, not just any cell. Specifically they state:

Unlike the random or "stochastic" model dominant in cancer research, which holds that nearly any cancer cell has the potential to form a tumor, the cancer stem cell model is one of a hierarchical organization, with the pluripotent cancer stem cell sitting ready and able to amass all of the components of the original tumor.

It's also thought, with some experimental evidence to support it, that CSC pluripotency allows these cells to adapt and to resist chemotherapy, radiation therapy, and even current molecularly targeted therapies. If true, then these treatments may not harm the most lethal tumor cells, those that can lead to a recurrence with the production of a new set of progenitors.

Despite numerous studies published in the last 16 years that identified CSCs for different cancers—including colon, brain, pancreatic, and breast cancer—the consensus among researchers seems to be that the evidence is strongest for the first cancer in which a population of tumor-initiating cells was discovered, acute myeloid leukemia (AML), as well as for other blood cancers.

They also continue:

"The reason why it's so much stronger for hematologic malignancies is because hematopoiesis research goes back 40 or 50 years and it's very stem cell-based," said Dr. Jean Wang, a stem cell researcher at the University of Toronto. "Whereas in solid tumors, there's less of a foundation for identifying the normal cellular hierarchies and for [cell-surface] markers that identify different populations of cells like stem cells and progenitors."

The above comment has some merit but one must also recognize that the hematopoietic cells are fundamentally generated in a specific location, the bone, and there may very well be no such locations specificity for the many other cells we are considering. Nevertheless, we continue:

Even so, Dr. Wang believes the existence of CSCs is pretty well demonstrated for breast and brain cancers. But, she cautioned, "I don't know if it applies to all cancers. In a lot [of cancers] it does seem to apply. But most of the markers we have right now are still very rough." Despite the evidence for CSC-like cells in a growing number of cancers, the theory clearly has its skeptics, who point to problems such as shortcomings in the mouse xenograft assay and the variable specificity of the cell-surface markers used to demarcate a CSC from a non-CSC.

"I still feel that it's a concept yet to be proven," said Dr. Barbara Vonderhaar, who, along with colleagues in NCI's Center for Cancer Research, recently published a study identifying a population of CSC-like cells in estrogen receptor-negative breast cancer. "It's certainly a good idea, but it's only a hypothesis at this point. We still don't have definitive proof that cancer stem cells exist."

The CSC concept is "a work in transition," said Dr. William Matsui, from the Johns Hopkins School of Medicine, whose lab studies the role of stem cells in hematologic cancers. "To me, as a clinical person, the ideal model is one where you can find something that is going to work in humans. We're far from that."

What is then most interesting is the extended discussion regarding the CSC and melanoma specifically. They state:

One of the most well-known studies in the CSC literature came from Dr. Morrison's lab in 2008. Earlier studies had suggested that, consistent with the CSC model, there was only a rare population of cells from human melanoma tumors that, when injected into mice with compromised immune systems (called NOD/SCID mice), could form new tumors.

But in a study published in Nature (see Quintana et al), Dr. Morrison's team tweaked the common experimental approach: they used mice with immune systems that were even more impaired than NOD/SCID mice and waited longer to assess tumor growth. The result: approximately one in four randomly selected single cells taken from a human melanoma sample could form a tumor.

The results "made clear that estimates of the frequency of tumorigenic cells are far more assay-dependent than we realized," Dr. Morrison said. In addition to factors such as the status of the mouse's immune system in the experiments, he continued, "there are probably other variables that have a much bigger influence that we still haven't discovered." (In AML, it's worth noting,

use of more immunocompromised mice does not significantly increase the number of cells that can form tumors.)

Researchers from Stanford University earlier this month reported in Nature (see Boiko et al)that they had found a marker, CD271, that identified a somewhat unique population of cells that could produce melanoma in highly immunocompromised mice; anywhere from 2.5 percent to 41 percent of cells in their human tumor samples expressed the marker. In additional experiments using similar mice on which human skin was engrafted, only tumor cells with the marker could produce tumors and metastases in the mice. (In his lab, Dr. Morrison noted, the same marker did not differentiate tumor-forming from nontumor-forming cells.)

The fact that a fairly large percentage of the cells from the nine human melanoma tumors used in the study could initiate a tumor reflects a number of things, wrote lead author Dr. Andrew Boiko and colleagues in the Nature paper. Among them, an evolutionary type process selects for the survival of tumor cells that fail to normally differentiate during tumor development.

That might mean that a cancer stem cell isn't necessarily part of the original tumor, but due to various factors or influences—such as interactions with the immune system or hypoxia—certain tumor cells, maybe many of them, can activate a stem cell-like "program."

"I'm a firm believer that the microenvironment, the stem cell 'niche,' is every bit as important as the cell itself," Dr. Vonderhaar said. "I don't know if just any cell can become [a CSC], but there is a hierarchy of cells, and some may be able to function in a stem cell-like manner, and others may not."

The CSC field itself, Dr. Matsui noted, needs to move more quickly beyond just determining whether these cells can grow tumors on their own, "and ask what other properties they might have that contribute to clinical outcomes." Those might include their role in problems such as drug resistance or metastasis.

Some of the controversy surrounding CSCs "is a good thing," Dr. Matsui said, "because it forces us to be more rigorous in our work. The more rigor we can get in the research, the more clinically applicable all of the ideas are going to be."

The existence of CSCs in melanoma has been examined and as with many cancers is still open for discussion. However as we shall discuss later the CSC model does have certain interesting uses in the progression and metastasis of cancer.

For example:

Cell Proliferation: If we assume that the CSC is the dominant cell that proliferates and all others do not, albeit being cancer cells themselves, then the growth of PCa in terms of cells is complex but one can then more easily explain indolent PCa.

Metastasis: We know that metastasis occurred by lymphatic and hematological means. However PCa cells, non-CSC PCa cells may break loose and yet not result in classic metastasis. The issue then is one where it may be necessary for the CSC to move by these means.

Many other such issues will arise and we discuss the CSC idea here and we return to it later in the work.

Now we can view the stem cells as shown below. There is a stem cell which can give rise to a new stem cell of ultimately a Post Mitotic Differentiated Cancer Cell. The PMDC cannot replicate, whereas the stem cell can. For metastasis it is thus necessary to send out a few stem cells, not PMDC cells.

7.1 THE STEM CELL THEORY

In an NIH report they define cancer stem cells as follows:

A consensus panel convened by the American Association of Cancer Research has defined a CSC as "a cell within a tumor that possesses the capacity to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumor." It should be noted that this definition does not indicate the source of these cells—these tumor-forming cells could hypothetically originate from stem, progenitor, or differentiated cells.

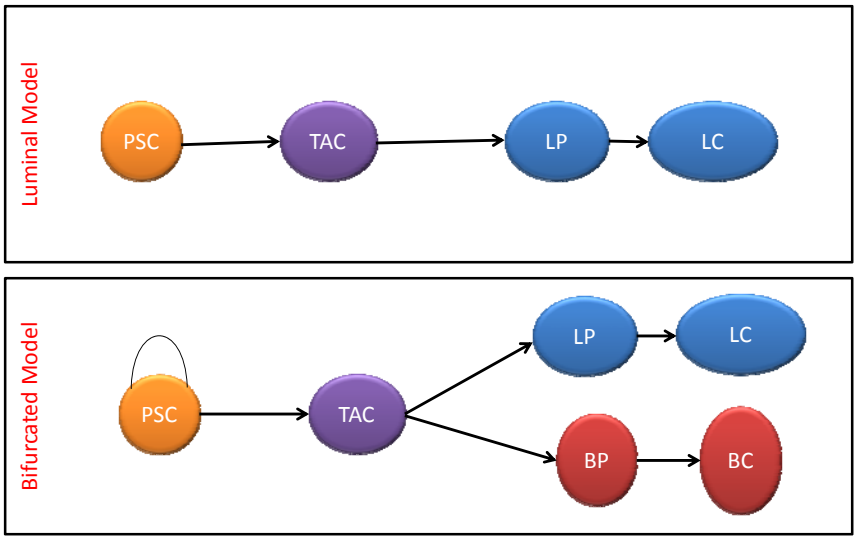
As such, the terms "tumor-initiating cell" or "cancer-initiating cell" are sometimes used instead of "cancer stem cell" to avoid confusion. Tumors originate from the transformation of normal cells through the accumulation of genetic modifications, but it has not been established unequivocally that stem cells are the origin of all CSCs.

The CSC hypothesis therefore does not imply that cancer is always caused by stem cells or that the potential application of stem cells to treat conditions such as heart disease or diabetes, as discussed in other chapters of this report, will result in tumor formation. Rather, tumor-initiating cells possess stem-like characteristics to a degree sufficient to warrant the comparison with stem cells; the observed experimental and clinical behaviors of metastatic cancer cells are highly reminiscent of the classical properties of stem cells.

The stem cell theory, and there seems now to be significant evidence of its validity in prostate cancer, is principally that the clonal theory has merit to a point but that the development is more complex and the cancer stem cell plays a critical role in fostering growth of the cancer cells, most of which has less aggressive a growth characteristic if any at all.

Lawson and Witte present a recent overview of this concept as applied to the prostate and PCa. Recent studies apparently indicate that the cancer stem cells, CSC, are necessary to sustain later stages of the development of the malignancy. Only a small subpopulation of the cancer cells, the CSC population, has a demonstrated ability to maintain the malignancy as well. Lawson and Witte present two theories of this CSC process. One is called the stochastic theory which is that all cells are equally malignant. The other theory, the one for CSC, called the hierarchical theory is that only the CSC has the ability to multiply. These two are graphically depicted below. The CSC or in this case the PSC, prostate stem cell, yields a TAC, or transition amplifying cells, then

yield progenitor cells, LP or BP, and then finally a luminal or basal cell. This is slight contrast to the Goldstein model. This model applies for both benign as well as cancer cells, at least as viewed by Lawson and Witte.



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Now if one looks at the CSC theory, then we see a CSC has progeny, and yet those progeny may not have the ability to multiply. Thus the explosive exponential growth of cancer is not as clear in a CSC model, because almost all of the progeny of the CSC are no reproducing progeny. Thus the growth models for a CSC based malignancy are more complex and are dependent on limited CSC reproduction and non-CSC reproduction. However the CSC model also argues for there being some CSC support for the progeny which are not CSC. The dynamics of cell growth then becomes quite complex here, for the stem cells replicate themselves at a slow rate but are replicating other cells at a higher rate. However the other cells do not replicate themselves they just go through a standard cell process. If the cells are benign then they go through apoptosis as seen in red blood cells and the skin keratinocytes.

As the Guardian states:

An emerging, although highly controversial, answer to this question is that cancer's immortality, too, is borrowed from normal physiology. The human embryo and many of our adult organs possess a tiny population of stem cells that are capable of immortal regeneration. Stem cells are the body's reservoir of renewal. The entirety of human blood, for instance, can arise from a single, highly potent blood-forming stem cell (called a hematopoietic stem cell), which typically lives buried inside the bone marrow. Under normal conditions, only a fraction of these blood-forming stem cells are active; the rest are deeply quiescent – asleep. But if blood is suddenly depleted, by injury or chemotherapy, say, then the stem cells awaken and begin to divide with awe-inspiring fecundity, generating cells that generate thousands upon thousands of

blood cells. In weeks, a single hematopoietic stem cell can replenish the entire human organism with new blood - and then, through yet unknown mechanisms, lull itself back to sleep.

Something akin to this process, a few researchers believe, is constantly occurring in cancer – or at least in leukemia. In the mid-1990s, John Dick, a Canadian biologist working in Toronto, postulated that a small population of cells in human leukemias also possess this infinite self-renewing behavior. ***These "cancer stem cells" act as the persistent reservoir of cancer – generating and regenerating cancer infinitely. When chemotherapy kills the bulk of cancer cells, a small remnant population of these stem cells, thought to be intrinsically more resistant to death, regenerate and renew the cancer, thus precipitating the common relapses of cancer after chemotherapy. Indeed, cancer stem cells have acquired the behavior of normal stem cells by activating the same genes and pathways that make normal stem cells immortal – except, unlike normal stem cells, they cannot be lulled back into physiological sleep.*** *Cancer, then, is quite literally trying to emulate a regenerating organ – or perhaps, more disturbingly, the regenerating organism. Its quest for immortality mirrors our own.*

We quote Lawson and Witte as follows:

Models of prostate epithelial differentiation. The traditional model for prostate epithelial differentiation proposes that PSCs residing in the basal cell layer give rise to intermediate, transit-amplifying cells that produce large numbers of terminally differentiated secretory luminal cells …. This model implies a linear differentiation scheme in which basal and luminal cells comprise one lineage and basal cells are essentially luminal cell progenitors …

This hypothesis is supported by the existence of cells of intermediate phenotype that express both basal- and luminal cell–specific cytokeratins in both fetal and adult stages of prostate development … Intermediate cells can also be identified in in vitro cultures of primary prostate epithelium … Several studies have also suggested basal cells can differentiate into luminal cells in vitro … Alternative theories for prostate epithelial differentiation propose basal and luminal cells may represent separate epithelial lineages … This is similar to prevailing models for epithelial differentiation in the mammary gland, a tissue that is anatomically and functionally analogous to the prostate …

Now there have been several others who have examined the stem cell model for PCa. Another of recent merit is that of Hurt et al. They summarize their work as follows:

Recent evidence supports the hypothesis that cancer stem cells are responsible for tumor initiation and formation. Using flow cytometry, we isolated a population of CD44+CD24- prostate cells that display stem cell characteristics as well as gene expression patterns that predict overall survival in prostate cancer patients. CD44+CD24- cells form colonies in soft agar and form tumours in NOD/SCID mice when as few as 100 cells are injected.

Furthermore, CD44+CD24- cells express genes known to be important in stem cell maintenance, such as BMI-1 and Oct-3/4. Moreover, we can maintain CD44+CD24- prostate stem-like cells as non-adherent spheres in serum-replacement media without substantially

shifting gene expression. Addition of serum results in adherence to plastic and shifts gene expression patterns to resemble the differentiated parental cells.

Thus, we propose that CD44+CD24- prostate cells are stem-like cells responsible for tumor initiation and we provide a genomic definition of these cells and the differentiated cells they give rise to. Furthermore, gene expression patterns of CD44+CD24- cells have a genomic signature that is predictive of poor patient prognosis. Therefore, CD44+CD24- LNCaP prostate cells offer an attractive model system to both explore the biology important to the maintenance and differentiation of prostate cancer stem cells as well as to develop the therapeutics, as the gene expression pattern in these cells is consistent with poor survival in prostate cancer patients.

Jordan et al characterize cancer stem cells as having three characteristics:

1. Self-Renewal: at the end of mitosis of the stem cell, either one or both retain all the characteristics of the parent. The stem cell goes through a mitotic doubling and when it does it always retains one or two stem cell daughters.

2. Capability to generate multiple lineages. This means that a stem cell can generate offspring which can become anyone of many cell types.

3. Potential to proliferate extensively. The cell can keep replicating, it has no limitation within reason and thus contains the elements ultimately for metastasis.

A normal stem cell may mutate to a cancer stem cell or a normal progenitor cell may morph back to a cancer stem cell.

As Delarbra et al state:

Although monoclonal in origin, most tumors appear to contain a heterogeneous population of cancer cells. *This observation is traditionally explained by postulating variations in tumor microenvironment and coexistence of multiple genetic subclones, created by progressive and divergent accumulation of independent somatic mutations.*

An additional explanation, however, envisages human tumors not as mere monoclonal expansions of transformed cells, but rather as complex tridimensional tissues where cancer cells become functionally heterogeneous as a result of differentiation.

According to this second scenario, tumors act as caricatures of their corresponding normal tissues and are sustained in their growth by a pathological counterpart of normal adult stem cells, cancer stem cells.

The statement starts with the accepted monoclonal hypothesis and then departs to a polyclonal alternative view. It retains the CSC, cancer stem cell, paradigm for solid tumors as well. In the context of HGPIN we see a change in the cells and we have heard the argument that they have made one or several of the unchangeable steps towards PCa. Thus using the CSC theory one would expect that it would be from one or several of these cells that PCa would arise. In

addition, we could assume that there is no unique pathway mutations or changes which result in PCa but a plethora of them. Simply stated, cancer is complex, it finds ways to migrate forward no matter what the path.

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A recent study by Deleyrolle et al has focused on the stem cell and its dynamics[112]. The reviewers state:

The method, published in the online journal PLoS ONE in January, may rev up efforts to develop stem cell therapies for Alzheimer's, Parkinson's and other diseases. It may also help get to the root of the cancer-stem cell theory, which puts forth the idea that a tiny percentage of loner cancer cells gives rise to tumors.

"Math is going to be the new microscope of the 21st century because it is going to allow us to see things in biology that we cannot see any other way," said Brent Reynolds, Ph.D., an associate professor of neurosurgery at UF's McKnight Brain Institute and a member of the UF Shands Cancer Center. "Stem cells and the cells that drive cancer may be as infrequent as one in 10,000 or one in 100,000 cells. The problem is how do you understand the biology of something whose frequency is so low?"

Inspired by a 2004 essay by Joel E. Cohen, Ph.D., of The Rockefeller University and Columbia University that described the explosive synergy between mathematics and biology, Reynolds and postdoctoral associate Loic P. Deleyrolle set out to build an algorithm that could determine the rate stem cells and cancer stem cells divide.

High hopes to treat or prevent diseases have been pinned on these indistinguishable cells, which are often adrift in populations of millions of other cells. Scientists know stem cells exist mainly because their handiwork is everywhere — tissues heal and regenerate because of stem cells, and somehow cancer may reappear years after it was thought to be completely eliminated.

This should be an interesting area to follow.

Nature has an interesting poster on the cancer stem cell, CSC[113]. The poster states:

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112 http://www.eurekalert.org/pub_releases/2011-01/uof-gfm012011.php

113 http://www.nature.com/nrc/posters/cancerstemcells/csc_poster.pdf

The concept of the cancer stem cell (CSC) has taken off rapidly over the past 10 years. CSCs are cells with properties that are similar to those described for tissue stem cells: self-renewal and asymmetric division resulting in the generation of daughter cells destined to differentiate, enabling the regeneration of a tissue. Initial research into the properties of CSCs was based on identifying and verifying markers of this subset of cancer cells.

However, most studies have now moved on to understanding the biology of CSCs and the cancers in which they maintain tumour growth, as well as how and why they are able to serially generate a tumour. It is thought that a key element regulating the biology of stem cells is their niche — cells and extracellular matrix that support self-renewal and survival. As we begin to understand the pathways that are crucial for the properties of CSCs, including signals provided by the niche, we will hopefully be able to effectively target this cell population. Linked to the identification of CSCs is the cell of origin.

These are cells that when mutated are able to give rise to a tumour. Although these cells may share properties with CSCs, in most cases it is not yet clear whether these cells are one and the same. This poster highlights some of the recent findings regarding the biology of CSCs and the identification of cell types from which cancers can arise.

As regards to prostate cancer they state:
In the normal prostate, epithelial cells with tissue-regenerating capacity that are Sca1+, CD49fhi, TROP2hi, CD44+, CD133+ and CD117+ (mouse) or CD133+, CD44+, CD49fhi and TROP2+ (human) seem to reside in the basal layer of the prostate. However, studies in mice indicate the existence of luminal cells with progenitor characteristics that can regenerate the prostate after androgen withdrawal.

As castration resistance is also a property of basal stem cells in the prostate, it suggests a complex cellular hierarchy. Studies in mice indicate that prostate tumours can arise after transformation of basal stem cells and luminal progenitor cells. A subset of cells that are CD133+, a2b1 + and CD44+ and have basal cell characteristics have been shown to be tumorigenic, but whether these cells can serially propagate tumours in mice has yet to be verified.

Again and interesting experiment can be performed:

1. Take biopsies from N men with HGPIN diagnosed on initial biopsies. Perform sampling from say 20 cores.

2. Wait 9 months, and rebiopsy, again with near saturation cores, 20+ .. There are three possible outcomes:

a. HGPIN remains
b. PCa has been determined
c. HGPIN regresses and only benign cells are left

3. The question is why did (c) above happen? What percent of the HGPIN have regressed? If the percent of HGPIN that have regressed equals the probability of having actually excised the cancer stem cell or cells, we can calculate this, then by chance we have removed the CSC from the HGPIN and this would affirm its existence by inference.

Now a similar article appears in Science which speaks to colon cancer and the cancer stem cell theory:

In normal colon tissue, intestinal stem cells (ISCs) that reside at the base of mucosal wells, named crypts, expand through mitosis and move upward toward the crypt tip. The cells then undergo cell cycle arrest and terminal differentiation, finally becoming the mucosal epithelium of the colon. In the recent study, the investigators identified in mouse ISCs a gene signature that was specifically marked by high expression of the ephrin type-B receptor 2 gene(Ephb2), which encodes a receptor tyrosine kinase, the leucine-rich repeat–containing G protein–coupled receptor 5 gene (Lgr5), which encodes a G-coupled protein receptor of unknown function, and ~50 other genes.

This gene signature also defined a specific population of stem-like cells at the base of colorectal tumor structures in mice that were morphologically similar to normal mouse intestinal crypts. The authors then similarly inspected tumor samples from 340 colorectal patients and discovered a 10-fold increase in the relative risk of recurrence in patients whose tumors displayed high expression of the human counterparts of the mouse ISC genes, relative to patients whose tumors showed low expression of these genes.

To test whether the mouse colorectal tumor cells with the ISC gene signature were cancer stem cells, the investigators isolated the cells and introduced them into an immunodeficient mouse model. The stem-like cancer cells demonstrated both a tumor-initiating capacity and self-renewal capability in vivo. *These findings pinpoint potential markers that may allow a clinician to predict a patient's future with respect to recurrence. These differentially expressed genes also may give rise to therapeutic targets that quell cancer stem cells.*

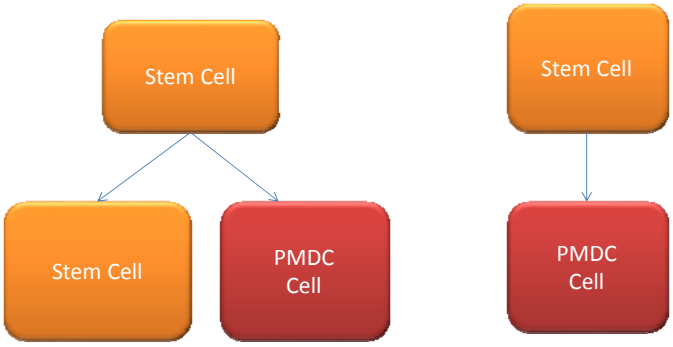
What is clear is that the CSC is becoming a viable model for understanding cancer at another level.

7.2 PROGRESSION AND REGRESSION

We first relook at the progression and regression dynamics. The key driver for the analysis herein has been the regression often seen in HGPIN. Knowing that most likely the methylation of GSTP1 has given rise to development of PIN we then ask what gives rise to its regression and why have the HGPIN cells themselves not only stopped growing but have disappeared. Again we have seen this in melanomas, and this is also the Rosenberg effect in certain sporadic cancer regressions.

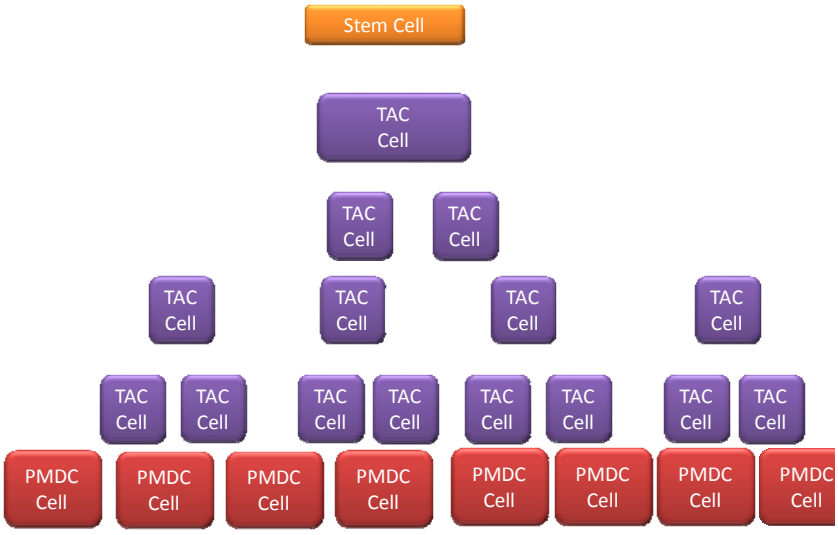
To look more closely we first return to the stem cell model for cancer which we developed earlier. The stem cell theory states that there are a certain number of cancer stem cells which in turn may replicate themselves but also create what are termed post mitotic differentiated cells.

Not really stem cells but cells which exhibit the phenotypic characteristics of a cancer cell. One of the questions one may pose is do these PMDC exhibit a different genotypic character as well or are they controlled by some epigenetic factors. We show these examples below;



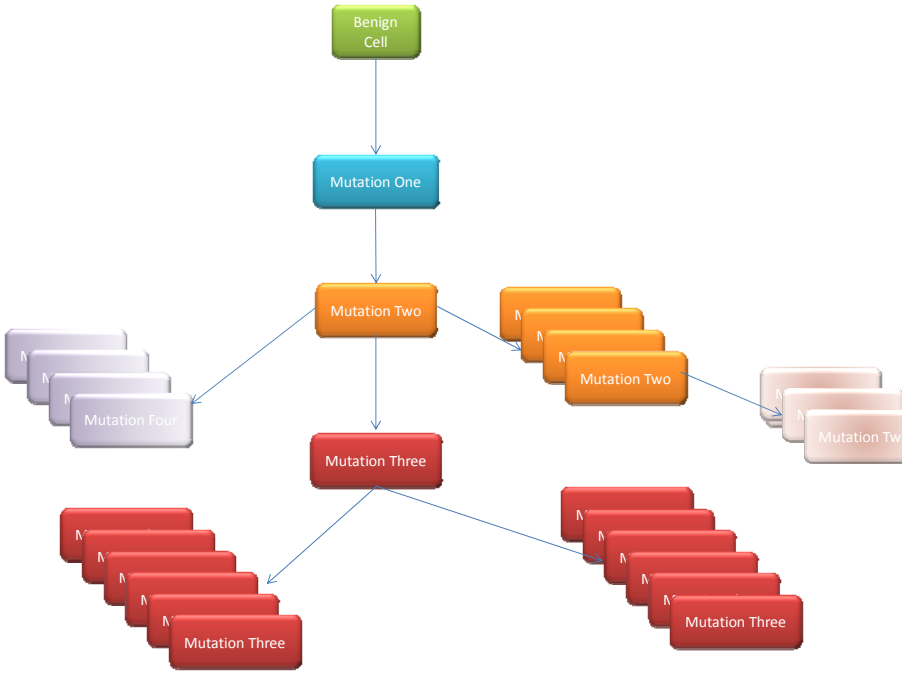
PMDC Cell: Post Mitotic Differentiated Cancer Cell

Now we can also see as Weinberg has noted (Weinberg p 419) that a progression may occur in a somewhat more complex mechanism as we depict below. Now from the stem cell arises Transit Amplifying Cells and then the PMDC.

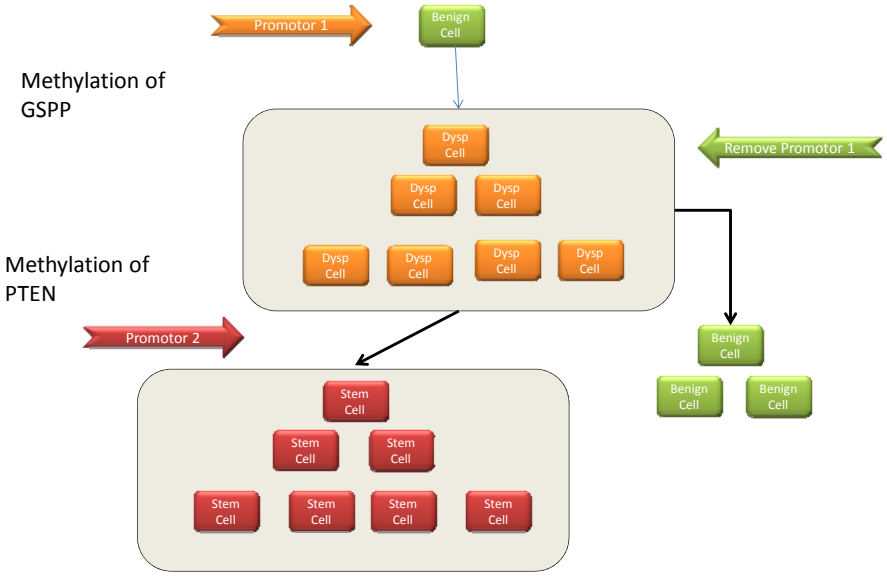


TAC: Transit Amplifying Cells

Now in reality there may be multiple genetic hits which give rise to the stem cell, the pluripotent self-replicating core of a cancer. The Figure below provides a generic profile, namely we may see many genetic changes, some leading to cancer as in mutation 3 below and others just wandering off into self-replicating cells but not with a malignant tendency.



Finally when we return to the HGPIN model we see the benign cell migrating to a dysplasia, say HGPIN, and then to a malignant cell, but then there is the regression back to a benign cell. The question is then; what pathway elements takes us one way and what elements take us back. And what happened to the dysplastic cells? Did they just die, apoptosis, or were they scavenged?



Wang and Shen have written a quite useful review of the cancer stem cell thesis for prostate cancer. There is no definitive conclusion but the review covers a wide path through what has been accomplished to date.

Recall as we have written before the cancer stem cell (CSC) model, and it is a model, hypothesizes that there are certain core cells which control the malignant growth of other cells and that the other cancerous type cells do not in and of themselves have the ability to continue to grow. In fact it could be concluded, although not part of the current theory, that removal of a CSC from a tumor, say the only CSC, would result in the apoptosis of the remaining cells. Namely a remission.

In contrast to the CSC model we have the clonal model which says that the cells have progressed through a set of pathway modifications that have resulted in a single cell which takes off and multiples and that the progeny have identical genetic makeup or further genetically modified makeup but all and equally malignant.

These are two fundamentally different views of cancer. One could also state that recent work with melanoma as we have discussed also posit that the CSC "communicates" to progeny to have them multiply and that arguably the loss of the CSC

There is a great deal of difficulty in identifying the CSC, usually attempting to do so via surface markers such as CD44 and the like.

Wang and Shen then discuss the controversy regarding the CSC concept. They state:

Much of the confusion in the literature arises through inconsistencies in nomenclature within the field. In particular, due to the wide use of xenotransplantation as a functional assay for CSCs, transformed cells that can initiate tumor formation in this assay are often referred to as CSCs in the literature. However, a tumor initiating cell (TIC) represents a different concept from that of a CSC, as TICs unquestionably exist within tumors and their identification does not by itself imply a hierarchical organization of a tumor.

Indeed, the majority of cells within a tumor could potentially possess TIC properties and nonetheless follow a clonal evolution model. Consequently, it is important to distinguish CSCs that have been strictly defined by their position and function within a lineage hierarchy in vivo from CSCs that have been identified as rare TICs in transplantation studies.

A similar confusion arises with respect to the cell of origin for cancer, which corresponds to a normal tissue cell that is the target for the initiating events of tumorigenesis. In principle, a normal adult stem cell could be a logical cell of origin for cancer, as it would retain the ability to self-renew and generate a hierarchy of differentiated lineages within a tumor. However, it is also possible that a cell of origin could correspond to a downstream progenitor cell or conceivably even a terminally differentiated cell that acquires stem cell properties during oncogenic transformation.

Our argument has been that the CSC may most likely exist and that it has undergone certain pathway changes and that as a result it may influence the growth of not identically genetically changed cells to multiply but not in and of themselves have the potential to multiply.

Wang and Shen continue:

The identification of normal cells that can serve as a cell of origin for prostate cancer is highly relevant for understanding the applicability of a CSC model, and is currently under intense investigation. The cell of origin may also have clinical significance, as in the case of breast cancer, distinct tumor subtypes have been proposed to originate through transformation of different progenitors within the mammary epithelial lineage. Thus, it is conceivable that there may be distinct cells of origin for other epithelial cancers, and different cells of origin may give rise to clinically relevant subtypes that differ in their prognosis and treatment outcome.

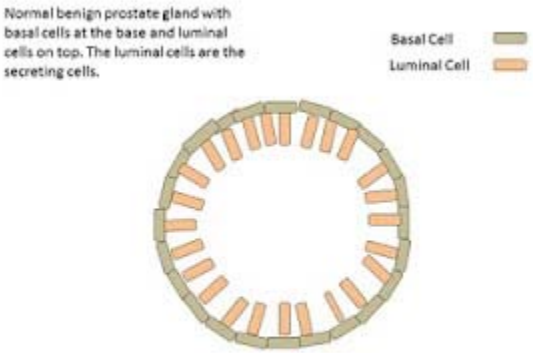
Thus there are either basal cells or luminal cells as the cell of origin. Goldstein et al in Witte's lab had developed a murine model demonstrating the basal cell as the cell of origin. However there may be strong issue regarding this model as applied to human prostate cancer. It represents a viable pathway but not necessarily the only. The issue is one of pathways as well as one of intercellular communications with debilitated pathways.

Now to follow the Wang and Shen model we have the following. Fist we show a normal prostate gland with basal and luminal cells.

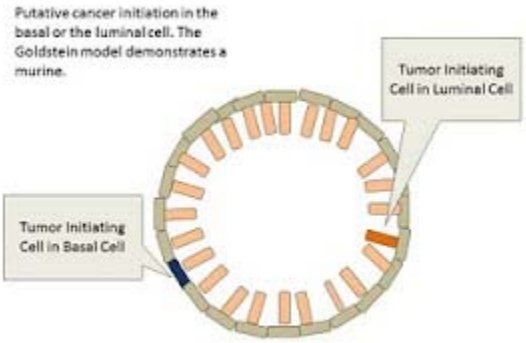
Then we show their view of a Tumor Initiating Cell in either the basal or luminal layer. The Goldstein et al murine model argue for the basal layer and there are others arguing for the luminal.

The Wang and Shen model is as follows.

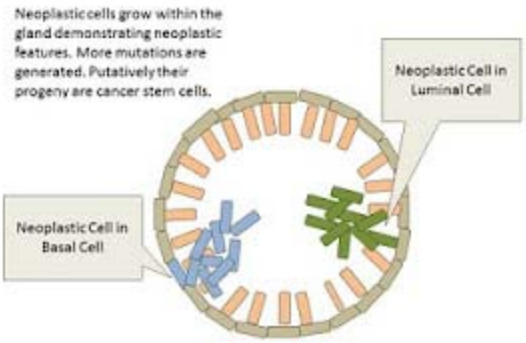
1. A normal prostate cell has both luminal and basal cells.



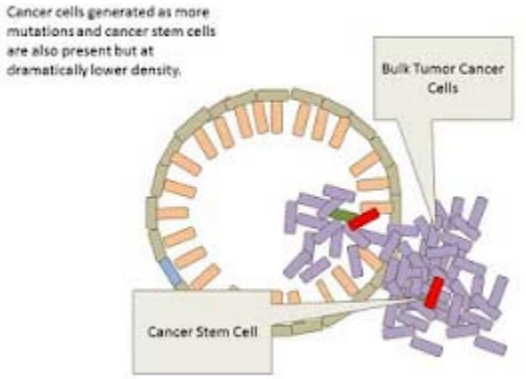
2. TICs may be formed in either basal or luminal cells.



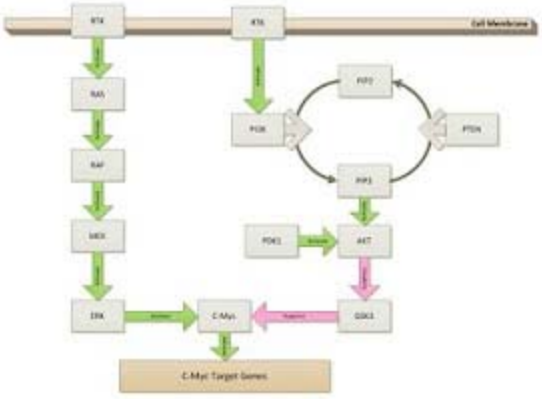
3. Neoplasia starts with intro acinar proliferation.



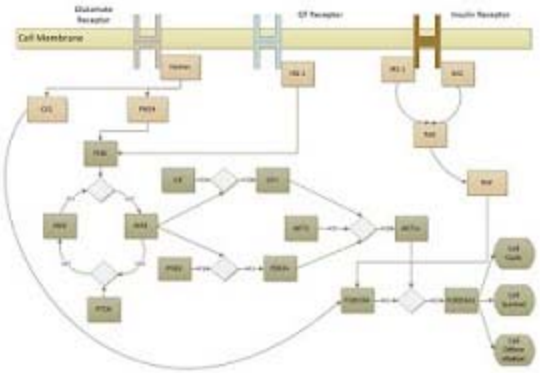
4. Carcinoma starts when it expands beyond the gland and starts up its own quasi-glandular structures.



Now what causes this? Genetic changes result in pathway changes. We show two pathways below. We lose PTEN and we may activate myc and other parts of the pathway control mechanism.



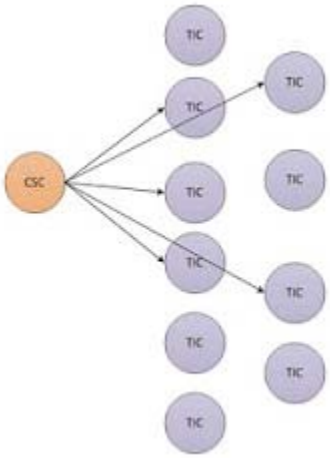
and the following pathway:



We now make a different argument. If there exists a true PCa CSC then perhaps one may putatively validate it as follows. The logic then is:

1. Assume a PCa CSC exists.

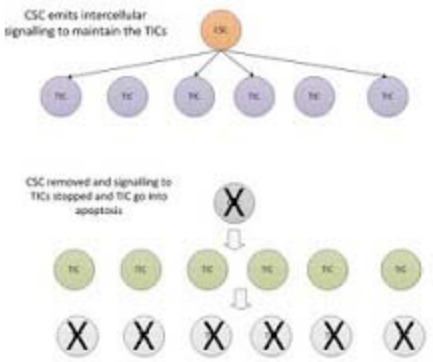
2. Assume that the PCa CSC replicates its CSC self at a low rate and is initially confined to the prostate gland.



3. Assume that the PCa CSC can influence the growth of TIC which themselves cannot sustain a malignancy. Specifically we assume that the TICs require the CSC for continued growth and further the CSC does so via cell growth as well as intercellular communications.

4. Now let us assume we have performed an 18 core biopsy on a 60 cc prostate gland and find histologically extensive high grade focal prostatic intraepithelial neoplasia. According to Wang and Shen they are most likely TICs and furthermore there may be a CSC somewhere so that eventually we see a PCa. There may be one or a few CSC in one or all of the glands yet we have no definitive marker to indicate as such.

5. Now assume we perform a second multi core biopsy on the gland and say do 22 cores in a 60 cc gland. This is the same gland but say 9 months later. We would arguably expect one of two possible outcomes. First that the HGPIN remains in place and possibly has expanded. Second that there was a CSC and the HGPIN had become classic PCa with say Gleason 2 or 3 at a minimum about the HGPIN clusters.

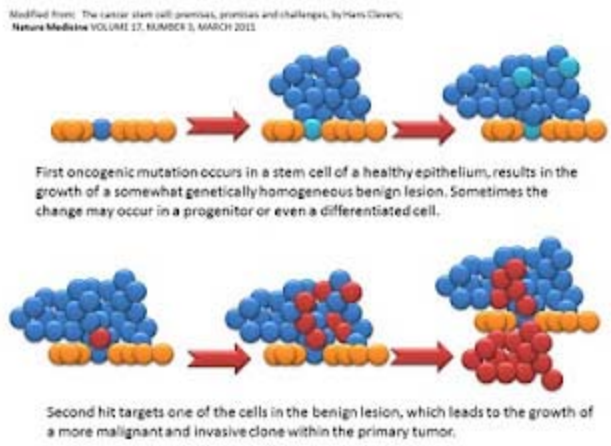


6. If however, we examine the cores and find no evidence of any neoplasia or PCa, namely the gland has totally reverted to benign histology, we may have a reasonable argument that perhaps the CSC was present initially, and it was somehow removed along with the HGPIN in the initial biopsy leaving the TIC alone behind. Thus the TICs requiring a CSC to survive go into an apoptotic state and are removed from the prostate. Perhaps.

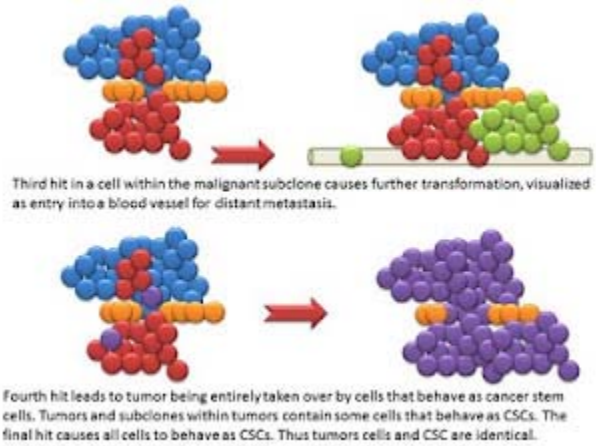
We have seen that specific situation occur and one could then argue that the Wang and Shen model for CSCs may be a viable model and further if such can be shown more extensively than we may have a basis for PCa progression.

There is an interesting article by Clevers in Nature Medicine which is an up to date review of the cancer stem cell issue. In light of the flurry of reports stating the wonders of having identified genes which appear in many tumors, prostate being the case, and my previous remarks that perhaps is the CSC is in fact existent, that then one should be identifying it and its genetic makeup as well as the dynamics of its pathways.

Now Clevers suggests a four step process, albeit with limited experimental evidence, but an superb start. It is as follows:



The above are the first two steps. Perhaps a dysplasia or neoplasia but with the kernel of a stem cell. This is the first "hit" theory. The epithelium starts to grow in a strange manner. Say a polyp in the colon or HGPIN in the prostate. Then we see a second hit and the formation of extraepithelial growth.



Then the third hit for the author and we see transmission via the blood stream. Then the fourth hit and the explosion from a few to almost all cancer stem cells.

Whether this is a good or bad model is yet to be seen. As Clevers states:

Central to the cancer stem cell (CSC) concept is the observation that not all cells in tumors are equal. The CSC concept postulates that, similar to the growth of normal proliferative tissues such as bone marrow, skin or intestinal epithelium, the growth of tumors is fueled by limited numbers of dedicated stem cells that are capable of self-renewal. The bulk of a tumor consists of rapidly proliferating cells as well as postmitotic, differentiated cells. As neither of these latter two classes of cells has the capacity to self-renew, the contribution of these non-CSC tumor cells to the long-term sustenance of the tumor is negligible.

The increased focus on the CSC is truly needed because if it is indeed a key paradigm in cancer then it and not large tumor masses should be examined. Clevers concludes with:

Epilogue: are CSCs and clonal evolution mutually exclusive?

To date, the CSC field has treated tumors as genetically homogeneous entities, by and large ignoring the fact that the observed tumor heterogeneity may result from underlying genetic differences. However, it is well known that most solid tumors show extensive genomic instability. Moreover, genetic defects in a large variety of molecules that are involved in the maintenance of the integrity of the genome are well-known drivers of oncogenesis.

Even in a disease like CML, so clearly driven by stem cells, clonal evolution can be seen at work when imatinib is administered: the malignancy becomes tumor-resistant through the emergence of clones that carry mutations in the target of imatinib, the BCR-ABL1 fusion gene75. And the progression of CML into ALL blast crisis is caused by the emergence of subclones that harbor inactivating lesions in the cyclin-dependent kinase inhibitor 2A (CDKN2A, also known as ARF) gene in addition to the BCR-ABL1 translocation76. The evidence for clonal evolution in the pathogenesis of cancer is so overwhelming that it appears inescapable that all models should be integrated with it.

The recent rapid advances in DNA sequencing are now allowing the global analysis of genomic changes of cancer cells. These analyses have confirmed many previously known common genetic alterations in cancer, and they have also revealed some new common mutations as well as unexpectedly large numbers of rare mutations. As a next step, this technology can be applied to chart genetic heterogeneity within individual tumors as well as between primary tumors and their local recurrences and metastases. ***It should thus be possible to map, in both space and time, the genetic evolution of a tumor.***

The last sentence is the most compelling. Cancer may be more than just a cellular disease, it may require the spatial domain as well. This is an exceptionally good review and should be a focus for future research.

7.3 MELANOMA AND CSC

As Fang et al state in their study of melanoma:

In approximately 20% of the metastatic melanomas cultured in growth medium suitable for human embryonic stem cells, we found a subpopulation of cells propagating as non-adherent spheres, whereas in standard medium, adherent monolayer cultures were established. Individual cells from melanoma spheres (melanoma spheroid cells) could differentiate under appropriate conditions into multiple cell lineages, such as melanocytic, adipocytic, osteocytic, and chondrocytic lineages, which recapitulates the plasticity of neural crest stem cells. Multipotent melanoma spheroid cells persisted after serial cloning in vitro and transplantation in vivo, indicating their ability to self-renew.

Furthermore, they were more tumorigenic than adherent cells when grafted to mice. We identified similar multipotent spheroid cells in melanoma cell lines and found that the stem cell population was enriched in a CD20+ fraction of melanoma cells. Based on these findings, we propose that melanomas can contain a subpopulation of stem cells that contribute to heterogeneity and tumorigenesis. Targeting this population may lead to effective treatments for melanomas.

Namely they contend that the CSC model does apply and that in addition the targeting of the melanoma CSC has potential application. They continue:

Indirect evidence supports the presence of melanoma stem-like cells.

First, melanomas show phenotypic heterogeneity both in vivo and in vitro, suggesting an origin from a cell with multilineage differentiation abilities. Melanoma cells retain their morphologic and biological plasticity despite repeated cloning.

Second, melanoma cells often express developmental genes.

Third, melanoma cells can differentiate into a wide range of cell lineages, including neural, mesenchymal, and endothelial cells.

They frequently exhibit characteristics of neural lineages. Melanomas from aggressive lesions can develop vessel-like structures and share with endothelial cells many matrix adhesion receptors such as h3 integrin or the cell-cell adhesion molecules MCAM, which are important for invasion and metastasis…

Then Fang et al conclude:

Our studies reveal that melanoma lesions can contain a subpopulation with stem cell properties and a fraction of more differentiated tumor cells. The adherent population established in vitro under standard conditions displays spindle to epithelioid morphology. In general, these cells reflect the stage of tumor progression from which they were initially derived…

7.4 FLOW, METASTASIS AND THE CSC

Why do zebras have stripes, asked Alan Turing just before he died from self-inflicted causes. The answer was, just at the time of Watson and Crick, because cells communicate with each other and turn genes on and off in an almost wave like fashion.

Evolving from that paper was the term Turing Tessellation. This means that cells produce proteins in an epigenetic manner and the proteins directly or as a result of the control they have on other molecules, communicate cell to cell, and this is what causes flower patterns.

In a recent paper by Roesch et al (2010) the authors show that in Melanoma the stem cell theory, namely that it is just one single cell that goes wild is proven wrong in melanoma. What happens simply is that the cells "communicate", a la Turing, and then when proteins which control

malignant cells start flowing they set off a chain reaction a la Turing and as we had shown in a paper a few years ago. The cells pop up elsewhere. The metastasis is not from the single stem cell escaping but from the proteins going wild.

One report in an interview with Roesch the author indicates[114]:

Scientists at The Wistar Institute offer a new explanation for the persistent ability of melanoma cells to self-renew, one of the reasons why melanoma remains the deadliest form of *skin cancer. The concept of the "dynamic stemness" of melanoma can explain why melanoma cells behave like both conventional tumor cells and cancer stem cells.*

The researchers write in the May 14 issue of the journal Cell that - contrary to other published reports - melanoma does not appear to follow the hierarchic cancer stem cell model, where a single malignant "mother cell" both reproduces to produce new mother cells and differentiates to produce the bulk tumor population. Instead, all melanoma cells equally harbor cancer stem cell potential and are capable of inducing new tumors. Their findings reveal the unique biology of melanoma, and suggest that melanoma requires a new therapeutic approach.

The article continue:

The present study arose when Roesch discovered a link between the potential of JARID1B to decrease proliferation of melanoma cells and control stemness. He decided to see whether JARID1B could be a marker of slow growing melanoma stem cells. Initially, the results were promising, he says. JARID1B-expressing cells were slow-growing (as stem cells often are), and rare, accounting for about 5 percent of the tumor population. "At this point we were really happy because we thought we had found a cancer stem cell marker," Roesch said.

But then, two unexpected results occurred. First, Roesch found that all melanoma cells were equally capable of initiating tumors in a mouse model, regardless of whether they expressed JARID1B or not. Second, he found that JARID1B expression did not conform to the traditional model of stem cell development - cells that expressed the gene could turn it off, and cells that didn't, could turn it on. In other words, the gene's expression was plastic, rather than stable. "Basically, our data suggest that every melanoma cell can serve as source for indefinite replenishment of the tumor," said Roesch.

Thus it is the cell to cell flow, the building and decaying of protein concentrations which set off the malignant melanocytes.

The Roesch paper summarizes the following key points:

1. *The H3K4 demethylase JARID1B marks a subpopulation of slow-cycling melanoma cells*
2. *The JARID1B+ subpopulation is required for continuous tumor maintenance*
3. *Cells can lose or gain JARID1B expression and do not follow a stem cell hierarchy*
4. *Tumor initiation is not necessarily linked with tumor maintenance*

[114] http://www.medicalnewstoday.com/releases/188783.php

This observation will undoubtedly change the "paradigm" in a Kuhn sense in viewing cancer. We have argued this for a few years now but it will take time for researchers to understand its implications in the full. For this we must thank Alan Turing.

As Roesch et al conclude in their paper:

The existence of a slow-cycling subpopulation is of high clinical importance because almost all current therapeutic regimens predominantly target the rapidly proliferating tumor bulk. Indeed, we observed that anti-cancer therapies in vitro (cytotoxic or BRAFV600E-targeted) uniformly result in an enrichment for JARID1B-positive cells. Thus, we postulate that targeting of the slow-cycling subpopulation, e.g., by inhibition of its H3K4 demethylase activity, in combination with a conventional debulking strategy could help eradicate all melanoma cells and increase the low therapy response rate in malignant melanoma.

If Roesch is correct then this represents an significant difference in the management of melanoma. Namely by recognizing the slow and fast cell rates. The fast rate is several mitotic changes per day and the slow rate is well over 4 weeks between mitotic change.

Roesch also states:

According to the traditional CSC concept, tumor initiation is regarded as an exclusive characteristic of CSCs. Thus, our finding that tumor initiation in immunodeficient animals is independent of JARID1B expression challenges the role of JARID1B as a classic CSC marker. In highly susceptible xenotransplantation assays like the one we used, the ratio of tumor initiating cells can increase up to 25% of unsorted cells. This suggests that either virtually every melanoma cell is a CSC because it can induce de novo tumors in xenograft assays irrespective of any known stem cell marker, or that melanoma is not hierarchically organized into subpopulations of tumorigenic and nontumorigenic cells and the CSC model does not apply

Specifically Roesch contends that melanoma fails the CSC test or if it does it means each melanoma cell is a CSC. The issue may also be partly a definition of CSC in the Roesch context.

In contrast we have the work of Perego et al who conclude:

Taken together, our data add to the existing evidence that human melanoma is a heterogeneous cancer composed of cells with different characteristics and suggest that melanospheres and their corresponding tumors in immunocompromised mice represent a reliable model to analyze melanoma biology. A better understanding of the fate of melanospheres inoculated in vivo, and their response to stimuli deriving from the tumor stroma, will offer the possibility to better understand the mechanisms driving melanoma formation and progression.

Namely Perego et al see that the melanoma CSC exists and furthermore melanoma is an amalgamation of disparate genetic cells all expressing different genes in an extreme view.

Perego initially contends:

Although there is no definitive consensus on the phenotype and frequency of CSCs in the majority of human tumors, much experimental evidence supports the contentions that many tumors of both epithelial and nonepithelial origin have operationally defined CSCs (cells able to propagate tumors in immunodeficient mice) and that the presence of these CSCs affects tumor biology.

Melanoma, one of the most aggressive human cancers with metastatic lesions resistant to conventional therapies, is highly heterogeneous and shows a high degree of plasticity. Recent evidence indicates that human melanomas contain different cell populations endowed with intrinsic chemo-resistance. Moreover, it has been shown that stem-associated markers such as CD133, CD166, and nestin are expressed by human melanoma. Although the nature and frequency of CSCs in melanoma is controversial, it is likely that the CSC model can be applied to human melanoma, and different surface markers have been proposed to define melanoma cells with tumor-initiating potential

That is what the authors do and their analysis lends strength to a CSC model in contrast to Roesch et al.

Other researchers such as Grichnik et al has stated:

Stem cells play a critical role in normal tissue maintenance, and mutations in these stem cells may give rise to cancer. We hypothesize that melanoma develops from a mutated stem cell and therefore residual stem cell characteristics should be able to be identified in melanoma cell lines. We studied three metastatic melanoma cell lines that exhibited multiple morphologic forms in culture and demonstrated the capacity to pigment

Namely they start with the CSC model, specifically a sustainable stem cell structure. One of the first questions they address is from whence does this melanoma cell come. To that issue they summarize:

The epidermal melanocyte is currently thought to serve as the origin for acquired benign and malignant melanocytic neoplasias. A stepwise ‘‘dedifferentiation’’ process has been proposed for the development of melanoma in which the mutated melanocyte passes through benign nevus, dysplastic nevus, melanoma radial growth, melanoma vertical growth, and metastatic melanoma stages. However, clinically, the majority of melanomas do not evolve in pre-existing dysplastic nevi and many evolve in normal appearing skin. An alternative theory has been put forth, suggesting that melanocytic neoplasias are derived from immature melanocytic cells.

Thus they question is classic step wise development from a normal cell which then undergoes mutations and results in a cancer cell. Namely they postulate a less linear form, namely starting from an immature melanocyte and thence a cancer cell.

They then make the statement:

Although much remains to be understood about normal melanocyte turnover, it is reasonable to assume that melanocytes in the human skin are normally replenished from a reservoir of immature cells. This is supported clinically by :

(1) disease processes such as vitiligo where differentiated melanocytes are destroyed, but when the disease is controlled, new melanocytes appear, and

(2) hair cycling experiments where hair bulb melanocytes appear to undergo apoptosis but are replenished by melanocytic cells in the outer root sheath, with hair regrowth.

Since cells with the capacity to proliferate and differentiate into melanocytes exist in the skin, their role should be considered in our models of melanocytic neoplasia.
The conclusion they draw from this set of observations is the existence of a melanocytic stem cell. Namely in vitiligo we have total melanocyte destruction but replenishment, inferring the supra existence of some stem like cell and the same with hair follicles and the normal apoptosis of the melanocyte.

Grichnik et al then poses their hypothesis:

Theoretically, if mutational events occur in stem cells giving rise to melanocytic neoplasias, then it is reasonable to assume that these mutated cells would attempt to continue to follow normal differentiation pathways until they accumulated sufficient genetic damage that this was no longer possible.

However, if the mutational events occur in mature melanocytic cells, the resulting tumor cells would be expected to be more aggressive than the original cell and expand in a homogeneous manner until additional mutations created new clones that would then repeat the clonal selection process.

We hypothesize that the mutagenic event that gives rise to melanoma occurs within a stem cell and not within a differentiated melanocyte. In the following experiments, we will demonstrate that melanoma tumors exist that have characteristics that are consistent with a tumorigenesis event based on a mutant stem cell.

Here they make a triple hypothesis: (i) that there exists some immortal stem cell, (ii) that mutations occur within the stem cell not the melanocyte, (iii) melanomas result from mutated stem cells and not mutated mature melanocytes. This becomes a powerful hypothesis if it can endure.

They continue:

There are several attractive features of the stem cell hypothesis for melanocytic neoplasia.

First, melanocytic nevi and melanoma would both have a common origin, being derived from a genetically defective stem cell. The severity of the defect in growth regulation would determine the extent of overgrowth; self-limiting defects would be benign, while nonlimiting defects would be malignant. An additional mutation could occur during the evolution of a benign neoplastic process, resulting in a malignant subclone, but this would not be required.

Second, the concept of ‘‘dedifferentiation’’ could be retired. Rather than melanoma ‘‘de-differentiation’’ from a melanocyte, the gross perception of de-differentiation could merely be due to the unmasking of a malignant stem/precursor cell with decreased ability to produce competent differentiated daughter cells.

Third, metastasis could be based on the more primitive stem cell biology, which may include its small size and differences in cell adherence.

Fourth, delayed onset and tumor dormancy may be more easily explained. Cells could exist unstimulated for long periods in the body, and then when environmental conditions are appropriate, the mutated stem cells could again give rise to TA/differentiating daughter cells and the tumor would become apparent.

Fifth, death from melanoma despite a strong immunologic response to melanocytic antigens can also be more easily explained. While the daughter cells might be easily destroyed by the immune system, the malignant stem-like cells might be overlooked

This description in a sense then redefines the stem call as a true stem cell of the melanocyte, rather than a stem cell of some cancerous mutation. Namely the underlying hypothesis of Grichnik et al is that there exist for melanocytes, true melanocytic stem cells, as we have in hematopoietic stem cells, cells which generate all other melanocytes, and it is within that reservoir of melanocytic stem cells that the damage is done. The issue however is we know that the hematopoietic stem cells come from within a reservoir in the bone. We do not have a similar reservoir for melanocytes which is obvious.

Grichnik et al conclude:

In summary, we believe that the stem cell model is consistent with our clinical knowledge of melanoma and normal biology and inconsistent with ‘‘de-differentiation’’. This stem cell model has diagnostic, prognostic, and therapeutic ramifications.

Now according to Liu et al (In Murphy) there are three models for this variation in melanoma and the CSC is one. Liu et al explains:

Tumor heterogeneity may be one reason for the differential responses to treatment and the survival of some, but not all. cancer cells following a given therapy. A number of models have been proposed to explain the phenotypic and functional heterogeneity that is found among the cells within a given malignant neoplasm, including melanoma.

These include:

(1) the clonal evolution model;

(2) CSC model; and

(3) the concept of cancer cell plasticity, also known as inter-conversion .

According to the clonal evolution model, tumor heterogeneity results from the continuous development of different clones as a function of cancer cell genomic instability . Clones with high potential for proliferation and survival are preferentially selected, and demonstrate enhanced growth and/or metastatic potential. While clonal populations are being established, the ancestral clone gradually disappears and the original genetics of the progenitor cell that initiated the cancer are lost.

According to this theory, drug resistance is due to the stochastic development of a clonal population bearing genetic and/or epigenetic aberrations. Therefore, evolution theory-based strategies to prevent cancer recurrence focus on the discovery of a putative drug resistance gene or an epigenetic alteration that could be targeted, and assume its presence in all cancer cells.

According to the CSC theory, cancer cells are hierarchically organized, with CSCs sitting at the top. In contrast to the clonal evolution model, CSCs have a relatively stable genome; thereby, maintaining more faithfully the genetic makeup of the ancestral founder cell. At the same time, drug resistance is an intrinsic property of these cells that is related to their functional status and not a result of selection during a specific therapy. Based on the CSC theory, drug resistance should be considered at the outset of any therapy, if tumor eradication is to be achieved.

According to this model, heterogeneity in the cancer cell population is dependent predominantly on differentiation of the CSC progeny and is somewhat irrelevant to resistance development, since these diverse cancer cells have limited self-renewal capacity and their long-term survival and colony formation is unlikely. This is a relatively well-recognized phenomenon in vitro, where individual cell cultures at a given time are less genetically similar compared with cultures of the same cell lines after multiple passages.

Cancer cell plasticity or inter-conversion describes the ability of cancer cells to -'switch" between different phenotypic states that may be associated with more or less aggressive behaviors and differential responses to treatment. The development of a population of cancer cells with limited self-renewal capability is conceptualized by a stepwise progression from CSCs. to transit amplifying cells (TA) to differentiated cancer cells.

7.5 CONCLUSION

The concept of a cancer stem cell for melanoma has both evidence and objections. The primary issue that it raises is one of suppression of a melanoma and what cell is the target. As we have noted one may suggest the target is the proliferation of aberrant or malignant cells. However to those who hold for a CSC then we are doing nothing more than debulking. The CSC may have not been affected at all, just the cells which it controls.

As Liu states:

Research on MSCs (melanoma stem cells) suffers for the same limitations as those experienced in other tumor models. It is also hampered by the high plasticity of melanoma, its unpredictable behavior and its unique resistance to current therapeutic modalities. As other aspects of cancer biology are being discovered, including "driver" mutations that promote tumor growth and immune system interactions responsible for tumor survival/rejection, it is becoming increasingly clear that combination therapies likely represent the best approach disease eradication. It is possible that another level of complexity should be added … Future chemotherapeutic strategies may target pathways that are less strictly associated with rapidly-dividing differentiated melanoma cell populations and more closely related to the metabolism of resting MSCs.

And Shackleton and Quintana conclude:

While the CSC model offers hope for improved treatment of some cancers by focusing research and therapies at rare populations of tumorigenic cells, it is apparent that not all cancers follow a CSC model. Although evidence from earlier studies suggested melanoma might follow a CSC model, recent studies in more permissive tumorigenesis assays argue strongly against this possibility. Effective treatments for melanoma will thus have to account for the fact that a high proportion of cells must be eliminated. The focus of research efforts needs to be firmly on unraveling the mechanisms that promote growth, metastasis and therapy evasion in the cells with tumorigenic potential that are so abundant in this disease.

Thus the MSC model for melanoma has some potential but the key factor is that it may drive one to a totally different direction when looking at pathway dynamics. That being said, if the pathway dynamics are the key to understanding control and management then it is essential that we understand the operative dynamics.

8 MELANOMA PROGNOSIS, MANAGEMENT, AND EXPOSURE

In this Chapter we address three specific issues:

First: We examine the current state of genetic markers in melanoma cells. As we note, there is a proliferation of reports on what genes now appear in melanoma cells. That on the one hand is the advantage of having such technology as the microarray and its downside. The issue we have spent a great deal of time on has been the pathway issue. Namely the understanding of what gene products are involved in homeostasis and what aberrations result in loss of normal cell control. We have not examined what causes the loss of certain gene products nor have we examined in details the dynamics of such cells. In addition in our discussion of the stem cell we have presented another option, namely a cell which controls other cells, directly or through the auspices of normal cells.

Second: We examine some of the current melanoma treatments. This is an exciting development because it uses understanding of the pathways and targets specific gene products, in the case BRAF. This may very well be a harbinger of things to come. We shall attempt to examine ways to control the cells vial later concepts.

Third: What causes melanoma? That is one of the overriding questions We have a great deal of literature indicating the impact of UV radiation, sun exposure, but what specifically happens. We thus examine another possible source, X-rays, and especially backscatter X-rays.

8.1 MELANOMA GENETIC MARKERS

Markers for melanoma are closely related to the pathways which a normal and a malignant cell progress through[115].

There is an almost daily set of new markers for a variety of cancers which are announced often with great fanfare. However the markers may or may not have any true meaning. We have discussed this in a prior posting and there we discussed the work by Venet et al as summarized by DeTours:

The signatures' prognostic potential can then be tested instantly in genome-wide compendia of expression profiles for hundreds of human tumors, all available for free in the public domain. Besides stem cells markers, signatures linked to all sorts of biological mechanisms or states have been shown to be associated with human cancer outcome. Indeed, several new signatures are published every month in prominent journals.

But such correlations are not all that they seem. The accumulation of signatures with all sorts of biological meaning, but nearly identical prognostic values, already looked suspicious to us and others back in 2007. It seemed that every newly discovered signature was prognostic. We collected from the literature some signatures with as little connection to cancer as possible. We found, for example, a signature of the blood cells of Japanese patients who were told jokes after

[115] http://www.mmmp.org/MMMP/public/biomap/listBiomap.mmmp

lunch, and a signature derived from the microarray analysis of the brains from mice that suffered social defeat. Both of these signatures were associated with breast cancer outcome by any statistical standards.

Namely DeTours and his co-authors seem to say that it is all too easy to get markers for almost anything. In the context of Dougherty and his work, one must have an underlying verifiable model for the process and then from that verifiable model one can attempt to ascertain what elements may have failed. Then and only then can one obtain truly prognostic determinants which in turn may lead to means and methods to reduce the disease state.

For example in just the recent past we have papers which have identified the following for melanoma:

6. MAP2K1 and MAP2K2 mutations (Nature Genetics, 2011)
7. MAP3K5 and MAP3K9 mutations (Nature Genetics 2011)
8. ACP5 (Cancer Cell 2011)
9. The following complex (Cell Oct 2011):
    a. A Sleeping Beauty screen followed by MuTaME analysis discovered putative PTEN ceRNAs
    b. The PTEN ceRNA ZEB2 regulates PTEN in a miRNA-dependent manner
    c. ZEB2 loss activates PI3K/AKT signaling and promotes cell transformation
    d. Attenuated ZEB2 expression is found in melanoma and other human cancers
10. SNPs as reported at (Nature Genetics, 2011):
    a. an SNP in ATM
    b. an SNP in MX2 and
    c. an SNP adjacent to CASP8 .
    d. A fourth locus near CCND1 remains of potential interest,

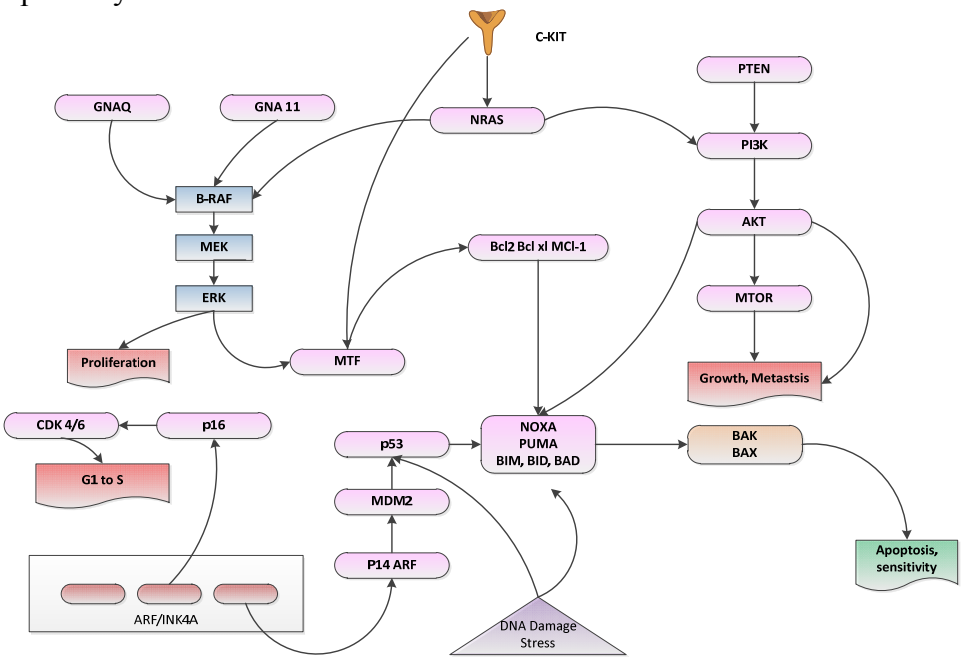
And the list goes on. As DeTours states, it may be all too easy to find aberrant genes, and even more so SNPs, independent of specific pathway models. And as I have argued, just within a pathway one may have a concern because it is also the intercellular signalling that is a concern as well. Even more so is the understanding of the process. Specifically:

5. A melanocyte may be normal until something happens. What is it that happens, does a SNP occur, why, when, and then what happens after that?
6. If a SNP occurs is that during the development of a DNA reading for protein generation or during cell replication. The opening of DNA for transcription may be the event which places the melanocyte at risk. If so then what is the risk process. Could it be radiation as suspected, or is it the next step in a Vogelstein like progression. Namely there may have already been

SNP damages and this one could be the final straw. Is it a micro RNA problem? The dynamics of this are essential.

7. Knowing pathways, is it possible to work backward and determine what the aberrant change or changes were? Pathway changes are reflected by their products.
8. What of the stem cell theory, must we look for the melanoma stem cell alone, and if so how can we identify it. The stem cell communicates, and that is a powerful mechanism to spread the cancer. How does it communicate and how is that related to the pathway.

Thus we look to understanding cancers in the context of pathways and then in the context of their intercellular pathways as well.



Understanding the pathway dynamics of melanoma has been progressing fairly well over the past few years. In a recent paper by Vidwans et al, the authors develop an interesting classification of melanoma based upon the specific pathway elements which may go awry. This is one of the first such classifications which goes beyond the classic morphological approach and even those using cell surface markers, This methods now looks at the cell dynamics and examines the malignancy based upon what specific pathway elements have failed. We show the pathway model used in the Vidwans paper above.

The now somewhat well understood B-RAF mutation, namely the V600E and discussed in the paper by Chapman et al:

Approximately 40 to 60% of cutaneous melanomas carry mutations in BRAF that lead to constitutive activation of downstream signaling through the MAPK pathway.[10,11] Approximately 90% of these mutations result in the substitution of glutamic acid for valine at codon 600 (BRAF V600E), although other activating mutations are known (e.g., BRAF V600K and BRAF V600R).

As Chapman et al state they have an inhibitor of the mutated B-RAF as follows:

Vemurafenib (PLX4032) is a potent inhibitor of mutated BRAF. It has marked antitumor effects against melanoma cell lines with the BRAF V600E mutation but not against cells with wild-type BRAF. A phase 1 trial established the maximum tolerated dose to be 960 mg twice daily and showed frequent tumor responses. A phase 2 trial involving patients who had received previous treatment for melanoma with the BRAF V600E mutation showed a confirmed response rate of 53%, with a median duration of response of 6.7 months.[16] We conducted a randomized phase 3 trial to determine whether vemurafenib would prolong the rate of overall or progression-free survival, as compared with dacarbazine.

As Bankhead states:

Patients with metastatic melanoma had an "astounding" 63% reduction in the risk of death when treated with an investigational agent that targets a mutation found in about half of the tumors, data from a large international trial showed.

Treatment with the BRAF inhibitor vemurafenib improved progression-free survival (PFS) by 74%. Analysis of six-month overall survival (OS) showed a 20% absolute difference between patients treated with vemurafenib versus dacarbazine.

Though follow-up is brief, the results already make a case for vemurafenib as the comparator for future trials of new agents for advanced melanoma, Paul. B. Chapman, MD, of Memorial Sloan-Kettering Cancer Center in New York City, said at the American Society of Clinical Oncology meeting.

"The median follow-up was only three months, yet the hazard ratio for death was 0.37 in favor of vemurafenib," Chapman said in an interview with MedPage Today. "That's an astounding difference that is almost never seen in oncology trials."

From 40% to 60% of cutaneous melanomas have BRAF mutations that activate downstream signaling through the MAP kinase pathway. About 90% of the mutations involve a specific substitution at codon 600 (BRAF V600E), Chapman and co-authors wrote…

The above demonstrates how understanding pathways we can target pathway drugs to mitigate the progression of the disease. However progression free survival is of limited duration. The cancer cell finds alternative paths to mutate. Thus the question is does one target one path after another as they progress or try a multi mix cocktail in hopes of preventing the development of any new paths. Is it possible, for example, to stop the transcription of melanocytes all together, and thus stop any and all expression so as to silence say all pathways.

In another piece Bankhead states the cost issues:

Vemurafenib has an estimated cost of $56,000 for a six-month course of therapy, and ipilimumab costs about $120,000 for four weeks of treatment. Both drugs also have potentially serious

adverse effects. In approving ipilimumab, the FDA cautioned that the drug has been associated with severe adverse effects that have included "severe to fatal autoimmune reactions."

The problem is that although the results are highly favorable for the short term, approximately six months, the long term is still questionable. It may be like imatinib and CML, namely there is a change in the cancer stem cells allowing a work-around of the blockage. Thus the costs would be considerable. Also the use of multiple drugs may as in leukemias result in “cures”. However the above costs, which may be at $20,000 per month of life extended, are excessive. The quality of life extended may not be the best and the drug while providing a “benefit” has not truly changed the end state, namely death of the patient. It has merely delayed the inevitable.

The issue of drugs, pathways, and targeting a sustainable remission is more than likely the target. As one has seen in many childhood cancers the goal of a sustainable remission is achieveable with cocktails of drugs and perhaps such may be the case here as well. Vidwans et al refer to their web site (see http://mmdm.cancercommons.org/ml/index.php/A_Melanoma_Molecular_Disease_Model) which provides a superb interactive asset for linking pathway elements, disease stage, trials and specific modalities for possible mitigation and control. The Table below is a modified version of the Vidwans table taken from their paper.

Melanoma Subtype	*Pathway*	*Gene/Biomarker*	*Diagnostic Technique*	*Potential Theraputic*
1.1	MAPK	BRAF	Targeted Sequencing	BRAF Inhibitor
1.2-1.3	MAPK	BRAF/PTEN	Targeted Sequencing and IHC	BRAF Inhibitor AND PI3K, AKT, mTOR inhibitors
1.2-1.3	MAPK	BRAF/AKT	Targeted Sequencing and copy number	BRAF Inhibitor AND AKT, mTOR inhibitors
1.4	MAPK	BRAF/CDK4	Targeted Sequencing and copy number / CGH	BRAF Inhibitor AND CDK inhibitors
2.1	c-KIT	c-KIT	Targeted Sequencing	
3.1	GNAQ/GNA11	GNAQ	Targeted Sequencing	MEK Inhibitor
3.2	GNAQ/GNA11	GNA11	Targeted Sequencing	MEK Inhibitor
4.1	NRAS	NRAS	Targeted Sequencing	MAPK, PI3K Inhibitor
5.1	MITF	MITF	Copy Number	HDAC Inhibitor
6.1	AKT/PI3K	PTEN	IHC	PI3K Inhibitor AKT Inhibitor mTOR Inhibitor
6.2	AKT/PI3K	AKT	Copy Number	AKT Inhibitor mTOR Inhibitor
6.3	AKT/PI3K	PI3K	IHC	PI3K Inhibitor AKT Inhibitor mTOR Inhibitor
7.1	CDK	CDKN2A	Targeted Sequencing	CDK Inhibitor HDAC Inhibitor
7.2	CDK	CDK4	Copy Number/CGH	CDK Inhibitor
7.3	CDK	CCDN1/Cyclin D	Copy Number/CGH	HDAC Inhibitor
8.1	P53/BCL	Bcl-2	IHC	
8.2	P53/BCL	p53	Targeted Sequencing	
9	TBD			

Now in contrast we have seen, as previously indicated, many papers where we have been presented with prognostic markers for melanoma and its development. Yet none seem to develop and verify them in the context of an underlying system model. The above mentioned work of Vidwans et al seems to be one of the first to commence that effort.

Melanoma Subtype	Pathway	Gene/Biomarker	Diagnostic Technique	Potential Theraputic
1.1	MAPK	BRAF	Targeted Sequencing	BRAF Inhibitor
1.2-1.3	MAPK	BRAF/PTEN	Targeted Sequencing and IHC	BRAF Inhibitor AND PI3K, AKT, mTOR inhibitors
1.2-1.3	MAPK	BRAF/AKT	Targeted Sequencing and copy number	BRAF Inhibitor AND AKT, mTOR inhibitors
1.4	MAPK	BRAF/CDK4	Targeted Sequencing and copy number / CGH	BRAF Inhibitor AND CDK inhibitors
2.1	c-KIT	c-KIT	Targeted Sequencing	
3.1	GNAQ/GNA11	GNAQ	Targeted Sequencing	MEK Inhibitor
3.2	GNAQ/GNA11	GNA11	Targeted Sequencing	MEK Inhibitor
4.1	NRAS	NRAS	Targeted Sequencing	MAPK, PI3K Inhibitor
5.1	MITF	MITF	Copy Number	HDAC Inhibitor
6.1	AKT/PI3K	PTEN	IHC	PI3K Inhibitor AKT Inhibitor mTOR Inhibitor
6.2	AKT/PI3K	AKT	Copy Number	AKT Inhibitor mTOR Inhibitor
6.3	AKT/PI3K	PI3K	IHC	PI3K Inhibitor AKT Inhibitor mTOR Inhibitor
7.1	CDK	CDKN2A	Targeted Sequencing	CDK Inhibitor HDAC Inhibitor
7.2	CDK	CDK4	Copy Number/CGH	CDK Inhibitor
7.3	CDK	CCDN1/Cyclin D	Copy Number/CGH	HDAC Inhibitor
8.1	P53/BCL	Bcl-2	IHC	
8.2	P53/BCL	p53	Targeted Sequencing	
9	TBD			

8.2 Melanoma Treatments

In this chapter we examine several of the recent treatment modalities using the understanding of the genetic makeup of melanoma.

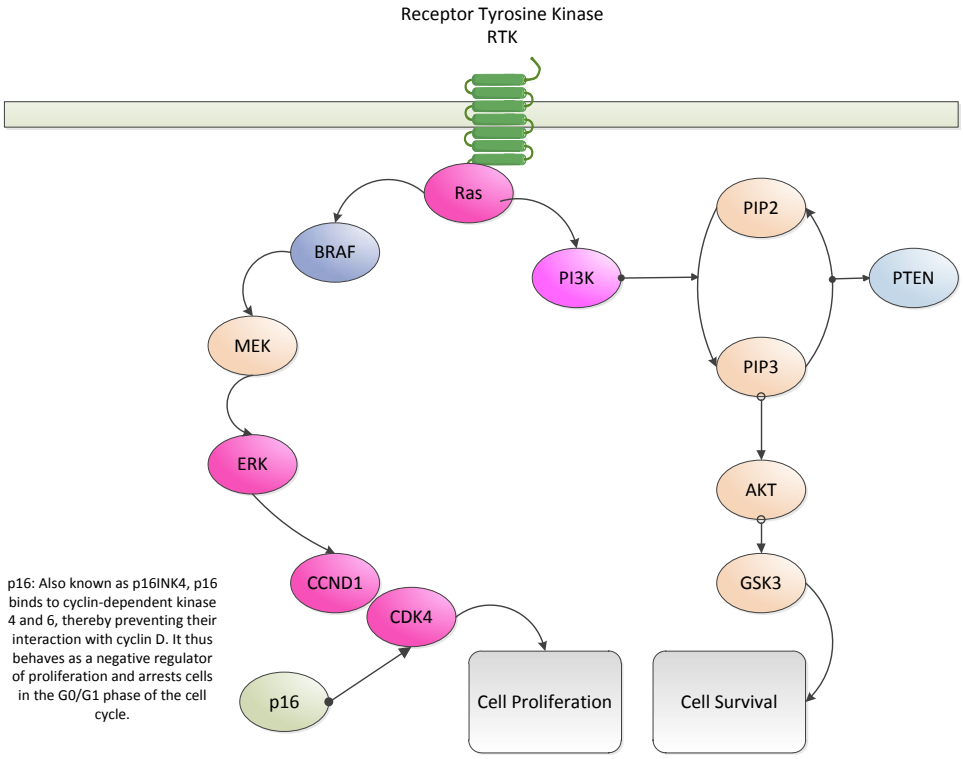
8.2.1 Recent Progress in Melanoma Treatment

There has recently been several pathway control mechanisms developed and tested and also an immunological approach deemed to be somewhat effective. We examine them here and also use them as suggestive of what else may be accomplished.

The current methods focus on two areas: (i) controlling aberrant pathways and (ii) using the immune response to control aberrant cells. It should be noted that in both cases we are dealing with the paradigm of a single but multiplying yet identical cancer cell. There is no hypothesis as regards to a stem cell or to the fact that the cancer may be multi-clonal.

8.2.2 Aberrant Pathway Control

We now examine the aberrant pathway approach. First let us consider the pathways that control a single cell. We show them below:



The above shows two results; cell proliferation and cell survival. They are two characteristics of a cancer. Namely the cell replicates and it does so in an almost immortal manner. The changed cell then starts to take over where other functional cells have been and the result is an unstable and ultimately deadly takeover of the human. Thus the two pathways are but a few of the many we will discuss at length. Yet the key point is that in examining melanoma it has been discovered that there is a specific mutation in the B-RAF gene that activate the MEK pathway. Activating that pathway creates a situation where we have an uncontrolled growth.

The growth factors activate the RTK kinase which activates the RAS which activates a B-RAF which overexpresses its product and this over-expression is what drives the proliferation

pathway. It is this single gene and its protein expression which causes the problem in 60% of the cases.

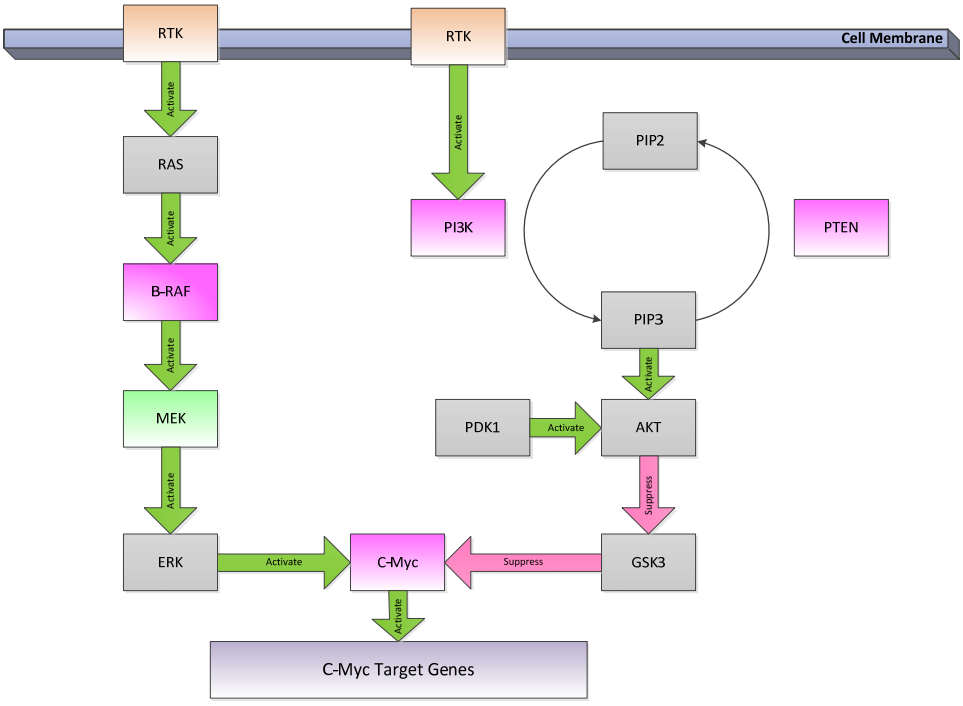
The cell survival is often controlled by PTEN and it is the loss of PTEN which results in the cancer cell immortality. The PTEN loss is comparable to the same issue we have seen in prostate cancer.

Key to aberrant pathway control is a simple principle. Namely, we base the approach on the observed fact that certain pathway control elements have been changed as a result of a change in the underlying gene. We will show that in the current well known example of B-RAF that the underlying gene of B-RAF has been mutated and it the resulting B-RAF protein which has allowed the pathway to be turned on permanently.

Thus the putative solution is to turn off the protein by targeting it with a drug which will pass the cell membrane and bind to the protein and inactivate it. A simple approach based upon an established fact. As we shall see there are two immediate issues: (i) only about 50% of the melanoma patients have the mutation, and (ii) the drug lasts for a relatively short time. It is similar to the effect that imatinib has on CML, a temporary regression and then a return.

As we shall see the possible solution may be multiple drug therapies targeting other pathway elements.

Now another way to view the pathways is shown below with the prominent role of c-Myc displayed at a common point. Note here we have the common surface kinases and the impact of B-RAF as well as PTEN. PTEN can modulate the limited up-regulation of B-RAF but only to a degree. As we have seen in PCa the loss of PTEN functionality leads to very aggressive forms.



The above also presents alternative control elements for possibly melanoma or frankly many other cancers. Specifically Smalley and Flaherty (2009) had suggested these pathway elements focusing on B-RAF, AKT and PI3K. One could also focus directly on the genes through a suppression mechanism but the technology for doing so is not yet available. Also there must be some specific targeting since we do not desire to target normal types of these products.

The control of aberrant pathways is conceptually simple.

1. Using a methodology such as microarrays, attempt to identify genes, or their expressions, which are present in the malignant cell. These are not unique and sometimes they are transient as well. The B-RAF identification is an example.

2. Develop a target molecule which can attach to and inactivate the aberrant gene or protein. In the current case of B-RAF they have deactivated the protein.

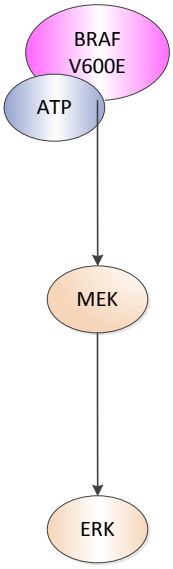
3. Test and use.

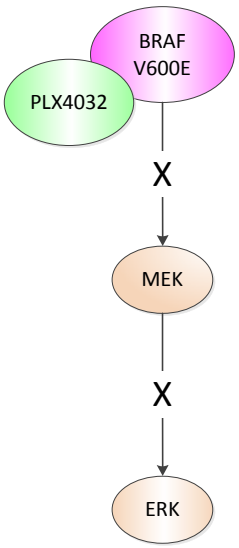
It may sound simple but the first step is potentially searching for a needle in a haystack and the second step can be as demanding. One may ask why not just block MEK or AKT just to stop everything. Assuming targets are possible the problem is it would do so for all cells and it would play havoc on the rest of the body. No blood cells, no hair, skin, and the like.

8.2.3 B-RAF control

The most recent one is the control of a mutated B-RAF, a variant of the RAF pathway. It was observed that there was a mutation in the B-RAF gene so that what was produced was a different B-RAF called V600E which had excessive up-regulation in almost 50-60% of all metastatic melanomas. The identification of this product then allowed for its targeting and suppression as a means to reduce cell proliferation. The results have been reported recently by the work of Chapman et al (2011) and Flaherty et al (2010). A review by Smalley and Flaherty (2009) had made suggestions on controlling both the BRAF as well as the AKT pathway. We will discuss that later. Recent work by Poulikakos and Solit (2011) has also presented both BRAF and MEK control, trying to avoid the loss of efficacy we discuss here.

Specifically, a drug now called Vemurafenib, or PLX4032, binds to the ATP activation site on the B-RAF mutation V600E and as such it blocks the overexpression of this protein and reduces the flow downward which we have shown causes ultimately an up-regulation of proliferation.

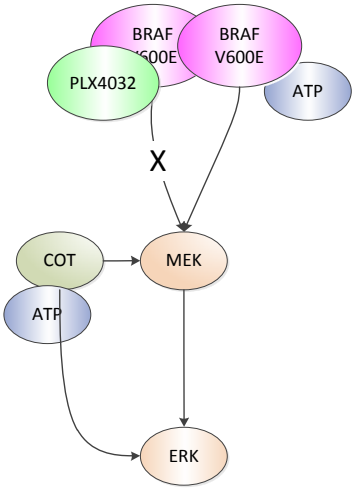




Note: PLX4032 replaces the activating ATP and deactivates the V600E BRAF.

Now we can also see that Vemurafenib can lose its effectiveness and there are several proposals for why this happens. We discuss a few here. From Solit and Rosen (2011) we show one of the possible ways in which resistance can occur. We discuss several of their conjectures in detail.

Below we depict the supposition from Solit and Rosen. Arguably this is what accounts for the mortality in the Kaplan Meir data they have from their trials.



From Solit and Rosen Fig 1: *"the overexpression of RAF1 or the activation of RAS as a result of RAS mutation or upstream activation of a receptor tyrosine kinase promotes:*

(i) the formation of RAF dimers. In cells expressing RAF dimers, binding of RAF inhibitors to one member of the dimer transactivates the other, nonbound member.

(ii) In such cells, PLX4032 does not inhibit MAP kinase signaling, which leads to drug resistance.

(iii) Alternatively, the overexpression of mitogen-activated protein kinase kinase kinase 8 (MAP3K8, or COT) results in RAF-independent activation of MEK and ERK and thus resistance to PLX4032.

(iv) The activation of upstream receptor tyrosine kinases may also cause resistance to PLX4032 by activating RAS, as well as by activating parallel signaling pathways, which results in diminished dependence of the cell on RAF signaling. PDGFRβ denotes platelet-derived growth factor receptor β, and RAS-GTP RAS in its active, GTP-bound state."

The paper by Solit and Rosen propose three reasons for loss of action of PLX4032:

(i) In melanomas with the BRAF V600E mutation, levels of activated RAS are too low to promote adequate formation of RAF dimers, and PLX4032 inhibits RAF activity and ERK signaling … This model is consistent with our observation that the introduction of mutant (activated) RAS

into cells with mutant BRAF causes insensitivity of the ERK pathway to the drug. This model suggests that increases in RAF dimerization (because of RAS activation or increased RAF expression) will cause ERK signaling to become insensitive to PLX4032 …

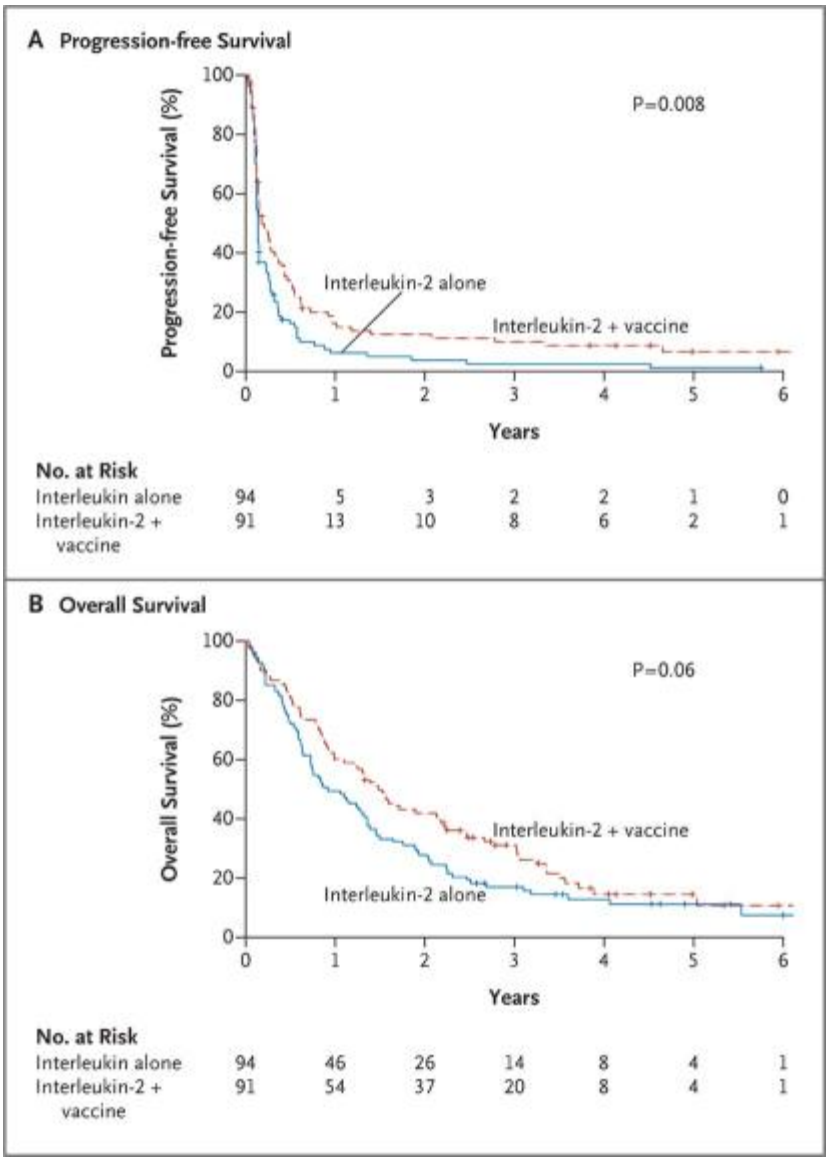
(ii) The findings of Johannessen et al. suggest another mechanism for the resistance of ERK signaling to RAF inhibition in cells driven by the BRAF V600E mutations. These investigators used a new technique −the introduction of a library of DNA constructs, each of which encodes a different kinase −into tumor cells with the BRAF V600E mutation to screen for kinases that confer resistance to RAF inhibition. Using this screen, they confirmed a previous finding: that overexpression of RAF1 confers resistance to RAF inhibition. [8] They further showed that the overexpression of mitogen-activated protein kinase kinase kinase 8 (MAP3K8, or COT), which phosphorylates MEK in a RAF-independent manner, can also mediate resistance to RAF inhibitors…

(iii) a third basis for acquired resistance, one in which the activation of other pathways causes the tumor cell to become less dependent on ERK signaling. In these tumors, ERK activation remains sensitive to the RAF inhibitor. Specifically, they report that platelet derived growth factor receptor β (PDGFR β), a receptor tyrosine kinase, is overexpressed in cellular models selected for RAF-inhibitor resistance in cell culture and in a subgroup of biopsy samples obtained from patients with progressing tumors. In the cell lines, PDGFR β overexpression was associated with resistance to the anti-proliferative effects of the RAF inhibitor, despite continued inhibition of ERK signaling in the presence of the drug.

8.2.4 Immunological Techniques

Rosenberg has for decades been examining the use of the immune system to attack cancer cells and he has done a great deal of work specifically on melanoma. The second thrust of the recent advances has been along these lines and Rosenberg has also been a contributor.

The first recent report is by Schwartzentruber et al (2011, NEJM) wherein, along with Rosenberg, they have used a vaccination of a peptide which can recognize melanoma cells and then by increasing the T cells via an interleukin infusion they found that the result was improvement in survival of metastatic melanoma patients. We show the results below from the paper.



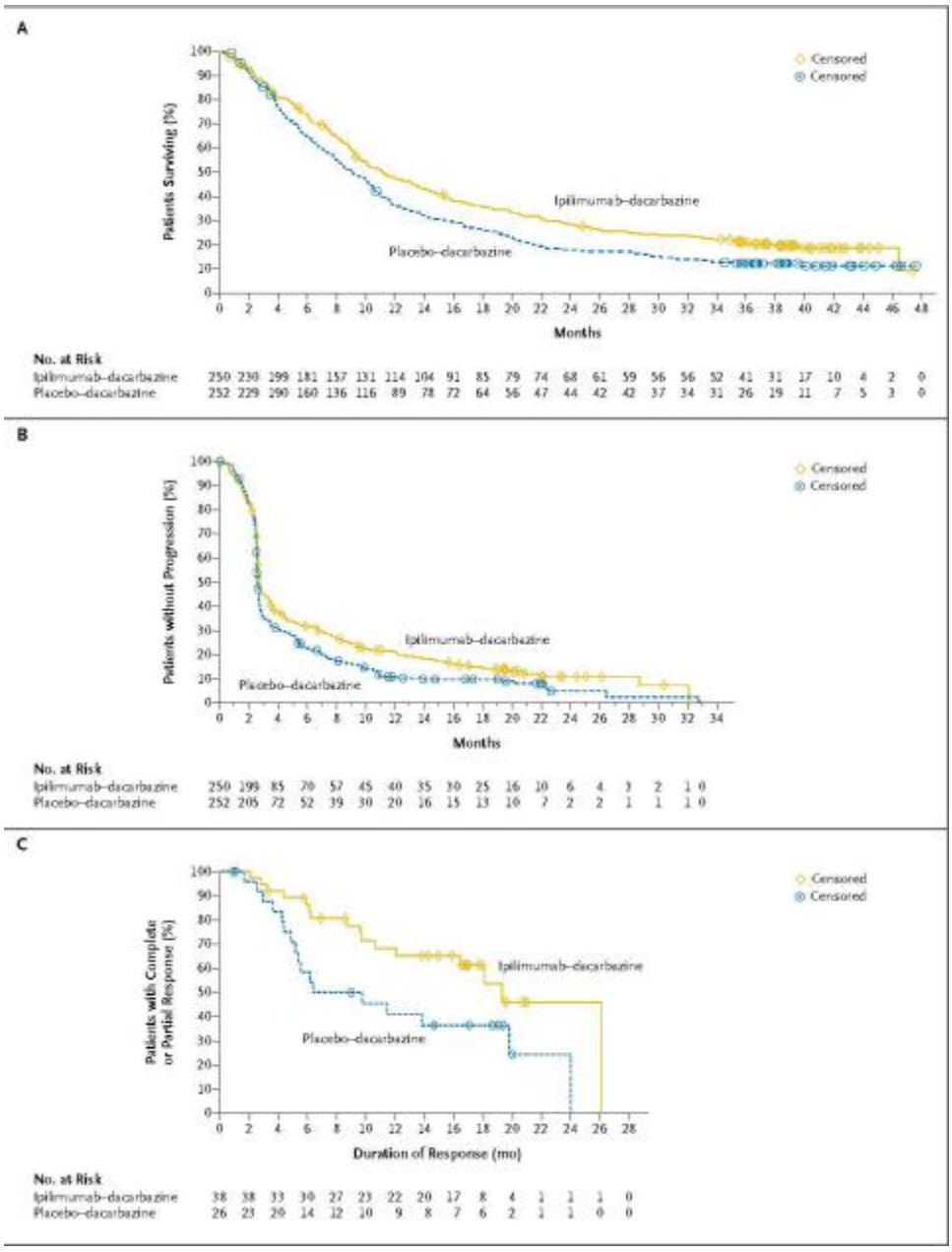
It should be noted that there is some improvement but still there is a very poor survival prognosis.

The second paper by Robert et al (2011 NEJM) uses another approach. They use a combination of a monoclonal antibody and a standard chemotherapeutic element. They state:

Ipilimumab, a fully human, IgG1 monoclonal antibody, blocks cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4), a negative regulator of T cells, and thereby augments T-cell activation and proliferation.

The second agent is dacarbazine. Decarbazine is a classic alkylating agent and has been used before with very limited results.

The data on survival is shown below:



Survival with the first approach after 36 months is about 38% and with the second approach it is about 45%. The interesting factor however with the second approach is the total remission in patients exhibiting total remission at the end of the study being almost 50%. Thus if total remission was exhibited it was sustained.

As the authors of the second study state:

Prolonged survival was noted among some patients who were followed for up to 4 years. In the ipilimumab–dacarbazine group, an estimated 28.5% of the patients were alive at 2 years, and an

estimated 20.8% at 3 years, as compared with an estimated 17.9% and 12.2%, respectively, in the dacarbazine group.
One can seem to state that the second approach was more effective than the first.

Possibly combining the approaches will be more effective and the current understanding is that they intend to examine those paths.

8.2.5 Considerations

The current efforts clearly show some significant advancement. However there are several key issues which must be clarified:

1. Is melanoma like colon cancer as described by Vogelstein or do we have a somewhat random set of mutations depending on the location of the lesion. Namely is melanoma really a disparate set of different sub-cancers.

2. Where does the melanoma stem cell fit in this paradigm? Stem cells have a problem because if they exist and are of the primary concern then perhaps we are just eliminating the TIC cells and not the CSC.

3. What of the Harahan and Weinberg model of an interacting environment? Namely what about the influence of the other parts of the body including the immune system? This has been a Rosenberg issue for decades and Harahan and Weinberg make a strong case for its consideration.

4. Is it necessary to develop a data base of aberrant expressions of proteins?

5. What about dealing with the gene itself? Why just the protein.

6. How can we identify these cells from say cell surface markers. That would enhance the ability to expand our understanding of the histology down to the expression level.

7. What genes have been changed and how? What was the change agent. We have argued elsewhere that it is radiation, ultraviolet and x-ray. But what of other factors.

8. As with other cancers, there may be a sequence of changes, and is MIS, melanoma in situ, one of the steps. Is MIS akin
to say HGPIN in prostate cancer or an adenoma in colon cancer?

There are many other issues which will evolve from this study. It represents a step in the forward direction but as has been seen each time we do this we see other new paths as unknown.

8.3 RADIATION AND THE MELANOCYTE

We now will look at the skin and the melanocyte and then examine the pathways which control melanocyte growth. Melanoma is a growing issue for individuals. As Rigel et al state[116]:

Melanoma is an increasingly important public health problem in the United States and worldwide. The incidence of melanoma has been increasing faster than that of any other cancer in the United States. Overall, melanoma incidence increased at an average of 4.6% annually from 1975 to 1985 and 2.7% annually from 1986 to 2007. Statistically significant increases are occurring for tumors of all histologic subtypes and thicknesses, including those greater than 4 mm. Invasive melanoma currently is the fifth most frequently diagnosed cancer in men and the sixth most frequently diagnosed cancer in women in the United States. In 2010, 68,130 newly diagnosed cases of invasive melanoma and 46,770 cases of in situ melanoma are expected. At current rates, the lifetime risk of an American developing invasive melanoma is approximately 1 in 58 overall and 1 in 39 for Caucasian men and 1 in 58 in Caucasian women. This contrasts dramatically with a lifetime risk of 1 in 1500 for Americans born in 1935.[4] Approximately 8700 people are expected to die from melanoma in the United States during 2010, accounting for 65% of all skin cancer deaths.

Thus as regards to x-rays and their ionizing effects it is essential to have a modicum of understanding of this cancer. This understanding must be able to go down to the cellular level including the molecular pathways as well as the specifics of cell growth and the normal transcription process of DNA.

8.3.1 Skin Anatomy

The skin is the largest organ of the body. A skin cell is about 30 μm in diameter and the top layer of the skin, the epidermis, may be 5 to 15 cells thick and this 150 to 450 μm in thickness, about 0.5 mm at the deepest. The skin is composed of:

1. Keratinocytes, the most abundant cells, which are always growing and migrating upward where they die off and fall off the person. The tow very top layers are the stratum corneum at the very outermost surface and then just below that is the stratum granulosom, the layer of dying keratinocytes.

2. Langerhans cells (4% of total):

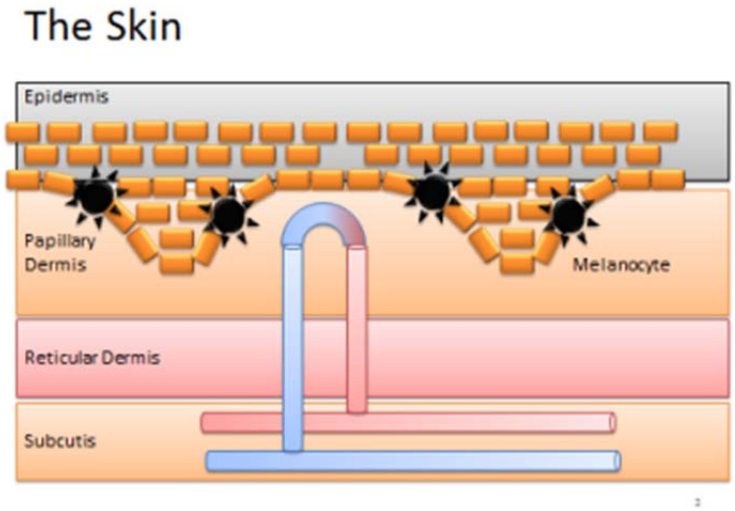
3. Merkel cells (<1% of total):

4. Melanocytes (3% of the total): The melanocytes remain at the basal layer of the epidermis and have long tentacles which spread upward to the upper layers and from these tentacles they emit the melanocytes, the pigment of the skin and the general pigment of a nevus. Any movement, up or down, from the basal layer, of the melanocytes is pathognomonic of a malignancy of some form. Stability of the melanocyte is the sine qua non of a benign cell. Unlike the keratinocytes,

[116] http://caonline.amcancersoc.org/cgi/content/full/caac.20074v1

which are reproducing and dying, the melanocytes are generally non-reproductive and stable. Their major function is to produce melanosomes.

The figure below depicts the characteristics of the skin. The papillary dermis is about 0.4 to 0.6 mm in thickness and contains blood flow both from below and within the layer itself. It abuts the epidermis. It is composed of many collagen fibers and blood and never fibers.

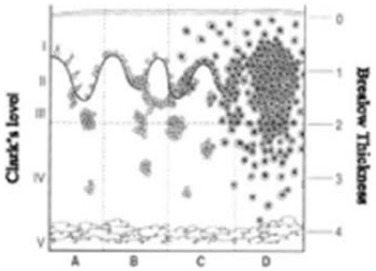


8.3.2 Melanoma and Pre-Dispositions

Melanoma is a very aggressive cancer and it is found I various forms. However the most common is superficial spreading melanoma which is a vertically spreading form before it develops a vertical spread and metastasizes. In contrast there is nodule melanoma and excessively aggressive form which starts with vertical spread and then attacks many organs. There currently is limited approaches with dealing with melanoma other than initial excision. Melanoma in situ is defined as melanocytes leaving their basal layer and moving into the upper portion of the epidermis. If excised there is a 100% cure rate for MIS. However if the melanocytes migrate downward and outward we then have the potential for deadly spreading melanoma and the cure rate drops accordingly.

The following shows the progression of melanoma:

Progression



Assessment of primary melanoma lesion growth. Clark's level (left) includes epidermal (I), dermal (II-IV) and subcutaneous tissue (V) compartments. Breslow thickness (right) is measured in millimetres from the top of the lesion to the deepest point of invasion.

Now the question is where does this come from; genetic, environmental, or what? There are a few genetic related causes. However, there are also predisposing conditions such as a dysplastic nevus syndrome which one can see raises the chance of the nevus becoming malignant. We will use the excellent overview by Dr. Ossia-Margarita Rosemarie Eichhoff in her thesis at Zurich to present the summary of these issues[117]. There are familial predispositions in some cases. Eichhoff states:

*A family history of the disease is identified in 10% of all melanoma cases …**The risk for individuals with a family history to develop metastatic melanoma is increased if there are multiple cases of melanoma in the family, multiple primary melanomas in a family member or early onset of disease in a family member**. Chromosomal analyses of melanoma-prone families have identified **two high-risk loci**. One is located at **9p21** and encodes the **CDKN2A** gene locus … Various mutations at 9p21 have been found in about half of all melanoma-prone families …. The absence of the mutation in other melanoma-prone families indicates the existence of other tumor suppressor loci not yet identified (Lesueur et al, 2008). The estimated worldwide penetrance for mutation carriers in CDKN2A is 30% by the age of 50 and 67% by the age of 8…*

***Another melanoma risk gene is located at 12p14** and encodes cyclin-dependent kinase 4 (**CDK4**… Only three families have been reported to carry mutations of CDK4 worldwide. Interestingly, all mutations occur in codon 24 … which is critical for Cdk4 binding to p16 (a CDKN2A locus gene product). Since the activities of Cdk4 and p16 both affect the same downstream effectors, it is not surprising that CDKN2A mutation-positive and CDK4 mutation-positive families present similar clinical characteristics …*

Thus there are at present just two recognized genetic predispositions for familial melanoma.

1. 12p4 encoding for CDK4

[117] http://e-collection.ethbib.ethz.ch/eserv/eth:1923/eth-1923-02.pdf

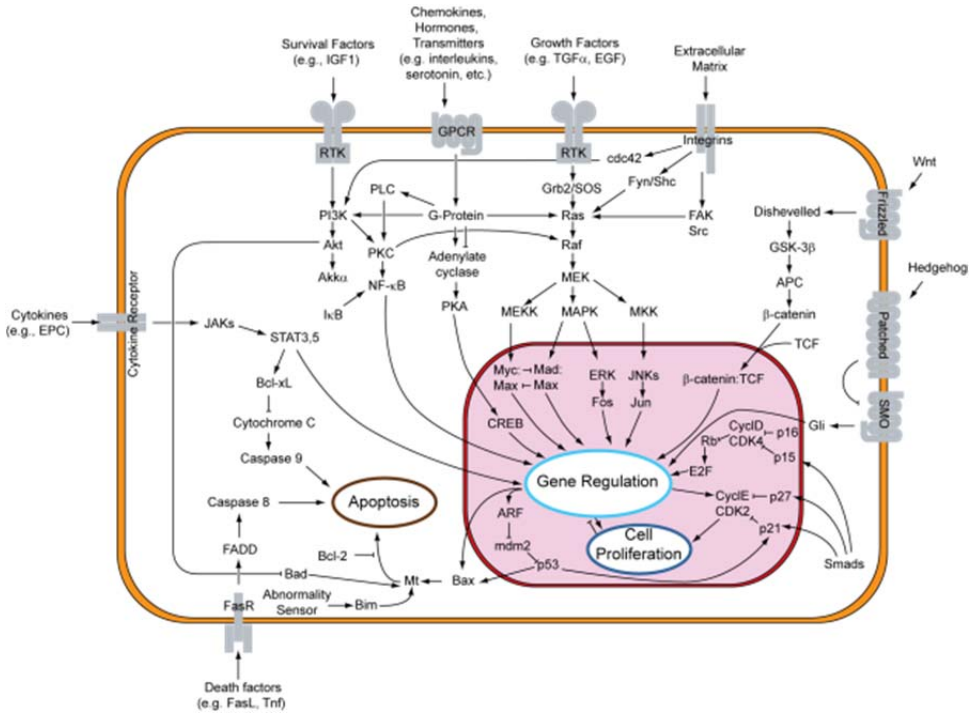
2. 9p21 encoding for CDKN2A

both being kinases.

8.3.3 Melanoma Pathways

We will now look at several specific genetic control paths and the ways in which their changes and alterations result in melanoma[118]. There are many such elements and pathways for many different cancers but we shall focus specifically on the one for prostate cancer. The Table below depicts a summary of prostate cancer impacting genes and genetic pathways. One must remember, however, the analysis of Dougherty, when looking at this Table. Just because a gene is present and expressing or absent and not expressing, one must understand the link of causality and not just the existence to draw complete conclusions.

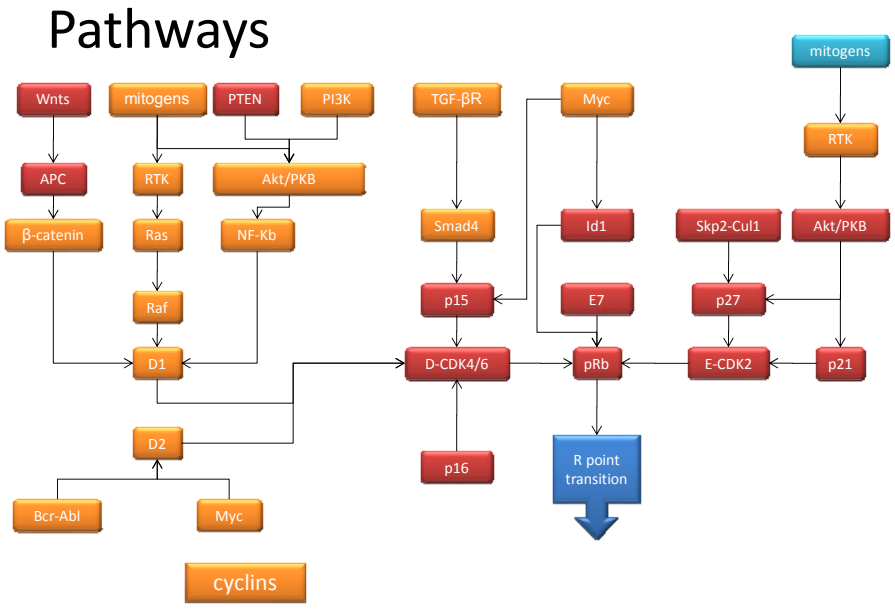
The following is a description of some of the many of the pathways found to be active in cancer cells[119]:



We can also look at some of these pathways in smaller detail as follows:

[118] There are many papers in this evolving field. The one by Bennett is also useful as a current update. There appears to be no generally accepted consensus as to what the ultimate pathways are. In fact this is a common issue across many cancers except a few, colon cancer being the most well understood on the pathway basis.

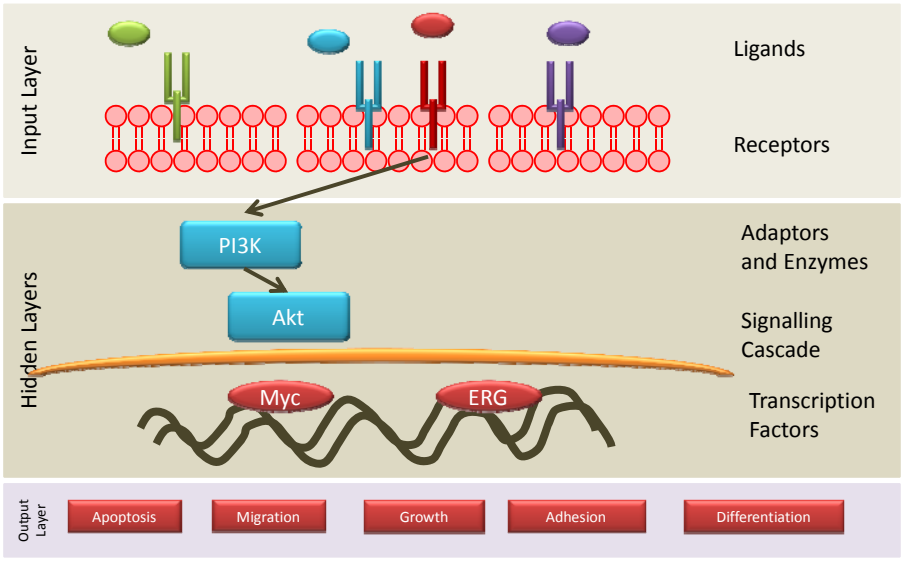
[119] http://en.wikipedia.org/wiki/File:Signal_transduction_v1.png



The R point transition control relates to the mitotic pathway a key to malignant growth. Many of these elements have been identified in the development of melanomas.

Pathways are a critical and fundamental issue regarding the homeostasis of cells. The elements in the pathways above are for the most part proteins derived from genes in the cell. If the genes are as they should be then the genes do what they should do, namely carefully fulfill its role. If, however, the gene has mutated for some reason, then the gene product may be changed and further the change may elicit additional changes. As we shall discuss later, changes may occur during the replication of the cell or during the basic process of transcription, going from DNA to RNA. In an ionizing environment, simple changes such as the switching of a single base pair resulting from some ionization may result in a change.

The following is from Weinberg and provides a good descriptive of what the elements in these pathways control. They deal with apoptosis or natural cell death, migration, growth, adhesion, and differentiation. Normal cells as they develop become good for one functional purpose within a single organ. Cancer cells have no productive purpose and just go everywhere eating up the environments where they land.



Before we discuss the possible changes, we will look at the key pathway elements as they are involved in melanoma. The very same pathway elements are involved in other cancers so what we are saying here for melanoma can apply in part for prostate cancer as well. Current research looks at the pathways and their aberrations for understanding what can happen in a normal melanocyte which would drive it to a cancerous state. We summarize referring to the thesis of Weinberg:

8.3.3.1 MAPK

*Somatic mutation of genes involved in the **mitogen activated protein kinase** (MAPK) pathway has been identified in a large proportion of melanoma cell lines and tumours. In normal tissues the MAPK pathway responds to cytokine activity by regulating cell growth, survival and migration via the transmission of a series of phosphorylation events to the nucleus. Extracellular signals are transduced through receptor tyrosine kinases (RTKs) by small membrane bound GTPases called RAS, which in turn activate a phosphorylation cascade of cytosolic kinases including RAF, MEK1/2 and ERK.*

Cytokine-mediated stimulation involves a diverse variety of RTKs such as epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR), c-kit and fibroblast growth factor receptor (FGFR). The hyper activation of these factors is frequently observed in a variety of cancers including melanoma.

8.3.3.2 RAS and RAF and BRAF

The following is a summary of the RAF genes:

ABL1 (ABL)	Translocation	Chronic myelogenous leukemia	RTK
ALK	Translocation	Anaplastic large cell lymphoma	RTK
BRAF	Activating codon change	Melanoma, colorectal, thyroid	RTK
EGFR	Amplification, activating codon change	Glioblastomas, non–small cell lung cancers	RTK
EPHB2	Inactivating codon change	Prostate	RTK
ERBB2	Amplification	Breast, ovarian	RTK
FES	Activating codon change	Colon	RTK

We look particularly at B-RAF which is frequently found in melanomas.

*The **RAS downstream target v-raf** murine sarcoma viral oncogene homologue B1 (**BRAF**) is mutated in 50-80% of melanomas most commonly with a single substitution (V600E) in its kinase domain. Kinase activity is regulated by the phosphorylation of T599 and S602 residues within the activation segment. Mutation of the valine at position 600 to a glutamate residue is thought to mimic the activating phosphorylation event and results in constitutive activation of the kinase. Since the $BRAF^{V600E}$ mutation is the most common in melanoma, it highlights the importance of the MAPK pathway regulation in melanocytic.*

Interestingly, 80% of benign nevi also express $BRAF^{V600E}$, and it has been demonstrated in nevi that expression of $BRAF^{V600E}$ drives senescence. This suggests that other control mechanisms are acting with the MAPK pathway in a cell context-dependent manner in order to regulate cell cycle checkpoints.

*Along with this finding it has been shown that the **incidence of mutated BRAF is high in vertical growth phase (VGP) melanoma but low in radial growth phase (RGP) melanoma** . Therefore, BRAF mutations alone are unlikely to be involved in the initiation of melanoma, but are rather involved in the hyper proliferative processes characteristic of melanocytic lesions. This further supports a cell context-dependency in which additional mutations, for example in Mc1R or PTEN, are necessary to drive malignancy.*

8.3.3.3 NRAS

*The **neuroblastoma RAS viral oncogene (NRAS)** is a GTPase activated by RTKs. When activated NRAS induces MAPK/ERK phosphorylation to drive cell proliferation. In addition, NRAS is also known to activate phophoinositide-3 kinase (PI3K) which inhibits apoptosis. Therefore, activating mutations in RAS lead to enhanced cell proliferation and survival. One such activating mutation in NRAS (Q61R) occurs most often in melanocytes of sun-damaged*

skin, with frequencies of 56% in congenital nevi, 33% in primary nevi and 26% in metastatic melanoma.

Interestingly, melanomas which show activating RAS mutations lack BRAF mutations and vice-versa, indicating that only one mutation in the MAPK pathway is necessary. It has been shown in mice that introduction of the NRAS (Q61R) mutation in a CDKN2A-deficient background promoted melanoma formation and metastasis to lymph nodes and other distal sites with 30% penetrance.

8.3.3.4 c-KIT

*The **v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene (c-Kit) is a RTK family member** and is activated by a specific cytokine known variously as stem cell factor, mast cell growth factor or Kit ligand. Activating mutations in the receptor's kinase domain increases the secretion of its ligand which in turn increases autocrine activation. It is reported that activation of c-Kit in a physiological setting is responsible for ERK2-dependent phosphorylation of Mitf and recruitment of the p300 transcription co-factor (CREB-binding protein), thus suggesting an important role for c-Kit in Mitf regulation and melanocyte development .*

Similar to MAPK pathway factors, mutations in c-Kit occur in melanomas derived from sun-damaged skin with a frequency of 28%. However, they also occur in melanomas derived from acral (36%) and mucosal (39%) tissues .

Interestingly, c-Kit receptor expression is reported to be down-regulated during melanoma progression and several studies have shown that either re-expression of c-Kit or exposure to stem cell factor in melanoma cell lines leads to their apoptosis. This suggests that the relevance of c-Kit activation to melanoma is more likely in malignant transformation of melanocytes than in disease progression.

However this contrasts with the observation that while constitutively activated c-Kit in mice leads to increased melanocyte migration, it does not increase cell proliferation or induce tumourigenic transformation.

8.3.3.5 PTEN

PTEN (phosphatase and tensin homolog deleted on chromosome 10) is a dual-specific phosphatase which can dephosphorylate phosphoserine and phosphotyrosine residues in target proteins (Myers et al, 1997). PTEN also functions as a lipid phosphatase by hydrolysing a phosphate residue in phosphatidylinositol 3,4,5-trisphosphate (PIP3) to produce phosphatidylinositol 4,5-bis phosphate (PIP2).

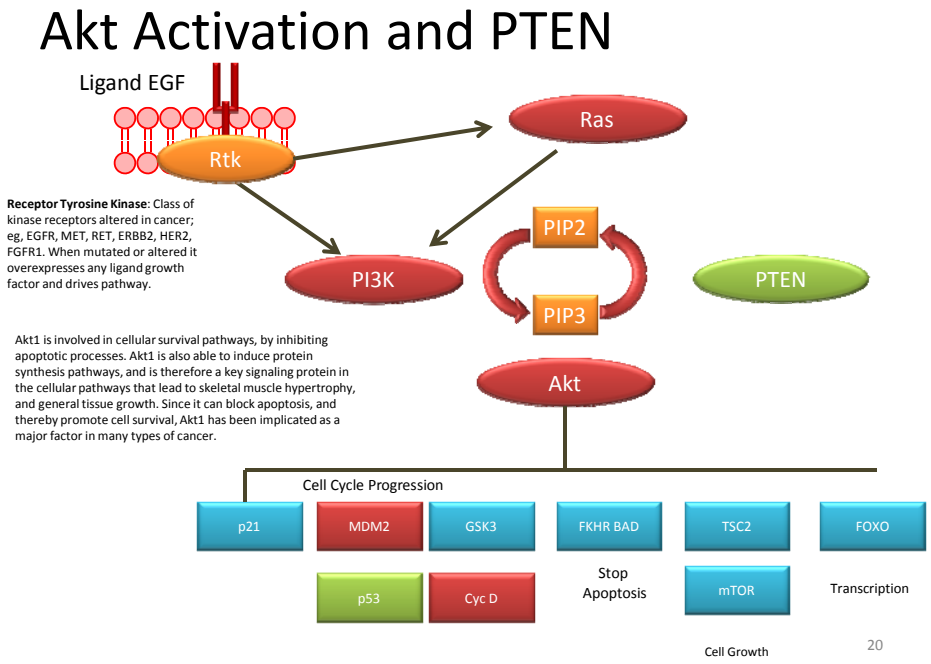
PIP3 mediates the phosphorylation and activation of serine/threonine protein kinases B (PKBα/β/γ, also known as: AKT1, AKT2, AKT3). By dephosphorylating PIP3 to PIP2 PTEN negatively regulates AKT kinase activity. Several studies have shown that AKT activity regulates the transcription of a wide range of genes, especially those involved in cell proliferation, apoptosis, and cell survival. Moreover it has been demonstrated that loss of PTEN leads to

decreased sensitivity to apoptosis through increased phosphorylated AKT . In contrast, forced expression of PTEN induces apoptosis through inhibition of AKT phosphorylation.

Therefore, the loss of PTEN and the activation of RAS are associated in the same pathway within the context of cell survival. The potential interaction between mutations in NRAS, BRAF and PTEN may play a critical role in melanoma development. Loss of PTEN function was detected with frequencies of 30% in melanoma cell lines and 10% in uncultured specimens. PTEN inactivation was found in only 8% of melanocytic nevi, but decreased levels of PTEN were found in 63% of primary melanomas (Tsao et al, 2003b). This indicates that loss of PTEN is related to melanoma progression and initiation. Recently it was demonstrated, in a mouse study performed by Dankort and colleagues, that an activating mutation in $BRAF^{V600E}$ led to the development of benign melanocytic hyperplasia that failed to progress to melanoma.

Strikingly, only the offspring from $BRAF^{V600E}$ mutated and PTEN-deficient mice developed melanoma with 100% penetrance of metastasis to the lymph nodes and lung (Dankort et al, 2009). This data shows how activated BRAF may cooperate with PTEN silencing to promote the development and progression of metastatic melanoma.

PTEN is a critical regulator which when closed down and inactive permits unregulated growth. We show the PTEN pathway in some detail below. Note the loss of PTEN activates Akt which sets in motion a set of other pathways. PTEN is often de-activated in prostate cancer as well as in many melanomas.



8.3.3.6 APAF1

The apoptotic peptidase activating factor1 (APAF1) is a downstream target of p53 which induces apoptosis by activating caspase-9. In malignant melanoma, about 40% of melanoma lesions show low expression of APAF1 due to loss-of-heterozygosity (LOH) and this inversely correlates with chemosensitivity in melanoma cultures (Fujimoto et al, 2004b; Soengas et al, 2001). Interestingly, loss of APAF1 is more frequently observed in metastatic melanomas than in primary tumours, and indicating that the significance of this factor may be in disease progression and not initiation (Fujimoto et al, 2004a).

8.3.3.7 WNT

The Wnt signal pathway is an evolutionarily conserved mechanism which is critically involved in both early development and cancer progression. It is currently thought that Wnt signal transduction follows at least three independent routs: the canonical (Wnt/βcatenin), non-canonical (Wnt/Ca^{2+}) and planar cell polarity (PCP) pathways. The Wnt family of proteins includes at least 19 different secreted factors which activate signaling by binding to Frizzled (Fzd) family receptors and their co-receptors including low-density lipoprotein receptor-related proteins (LRP), orphan tyrosine kinase receptors (ROR) and crypto. In humans, there are ten Fzds and two LRPs acting as receptors for Wnt signaling. This wide variety of Wnt ligands and receptors allows for a range of distinct cellular responses. Activation of Wnt signaling is regulated by a combination of secreted Wnt ligand availability and the expression of appropriate receptors.

For example, Wnt3a binding to a FZD/LRP receptor complex preferentially activates the canonical Wnt pathway. Alternatively, Wnt5a binding to a FZD/ROR2 receptor complex preferentially activates the non-canonical Wnt pathway. Furthermore, heparin sulphate proteoglycans (HSPGs) present on the cell surface and in the extracellular matrix are thought to play a role in stabilization and presentation of Wnt ligands to their receptors. This suggests that HSPG expression and glycosylation play an important role in mediating Wnt signal transduction. Finally, actin-based filopodia extension and cytoskeleton dynamics have also been shown to both influence and be dependent on Wnt signaling, thus regulating cell morphology and motility . There is also evidence for cross-talk between Wnt signaling and other pathways, including transforming growth factor-beta (TGF-β) and mitogen-activated protein kinase (MAPK) pathways.

Mutations in components of Wnt signaling are known to lead to various developmental defects. Similarly, in cancer the deregulation or hyper-activation of Wnt signaling is widely reported to drive tumor cell proliferation and metastatic progression . The following sections discuss the canonical and non-canonical Wnt pathways as well as their roles in cell-cell adhesion and motility during both melanocyte and melanoma development.

To summarize, we can say this of Wnt[120]:

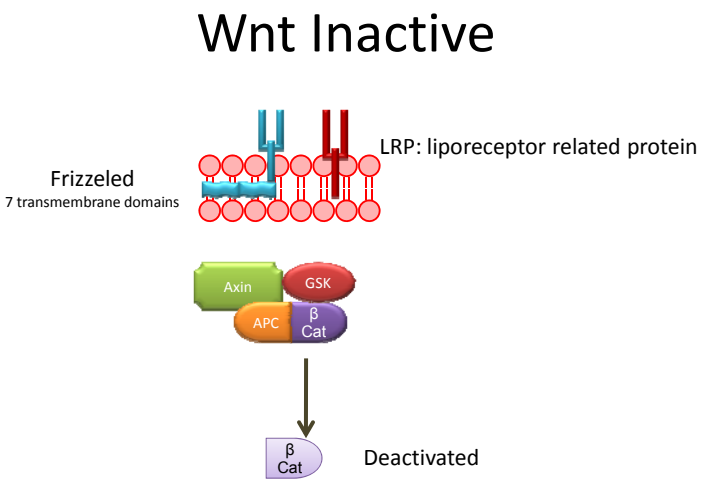
- The **canonical Wnt pathway** describes a series of events that occur when Wnt proteins bind to cell-surface receptors of the Frizzled family, causing the receptors to activate Dishevelled

[120] This comes from the "wingless" gene and thus the Wn prefex. The was related to discovery on fruitflies.

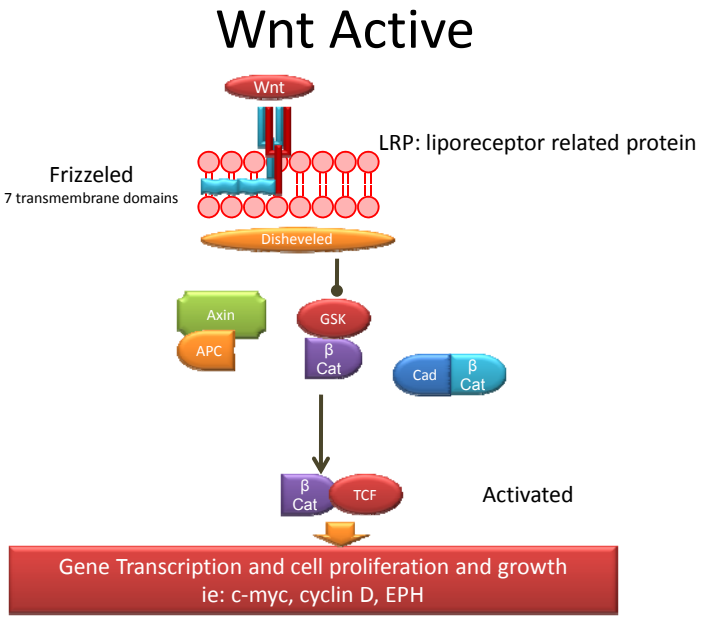
family proteins and ultimately resulting in a change in the amount of β-catenin that reaches the nucleus

- Dishevelled (DSH) is a key component of a membrane-associated Wnt receptor complex which, when activated by Wnt binding, inhibits a second complex of proteins that includes axin, GSK-3, and the protein APC
- The axin/GSK-3/APC complex normally promotes the proteolytic degradation of the β-catenin intracellular signaling molecule.
- After this "β-catenin destruction complex" is inhibited, a pool of cytoplasmic β-catenin stabilizes, and some β-catenin is able to enter the nucleus and interact with TCF/LEF family transcription factors to promote specific gene expression

Wnt also has several ways of acting. We show two here:



and when activated the following occurs:



The activation by Wnt results in cell proliferation and growth, and is often unregulated.

In another Zurich researchers thesis, Dr. Natalie Schlegel**,** it states:[121]

We contend that melanoma cells switch between two defined gene expression signatures, each underlying a distinct cell phenotype, which together drive disease progression. Presented in this thesis are the in vitro and in vivo experimental validations for this model, the investigation of the role of TGF-ß-like signalling, predominantly its role in growth inhibition, and the identification of Id2 as a gene involved in TGF-ß-induced growth inhibition response. After a literature review of genes identified to have phenotype-specific expression, we identified Wnt and TGF-ß signalling as drivers of the identified transcriptional signatures. By in vitro characterization of phenotypically opposed cells, we identified the two phenotypes as proliferative and invasive. As well as showing divergent proliferative and invasive behavior, cell types could be discriminated based on their growth susceptibility to TGF-ß and their capacity for vasculogenic mimicry.

Reduced susceptibility to the growth inhibiting effects of TGF-ß and the capacity for vasculogenic mimicry have both been associated with increased invasive and metastatic properties of melanoma cells. Our model suggests that both proliferative and invasive transcriptional signatures are important in disease progression and that each melanoma cell retains the capacity to express either signature given appropriate signalling. Our model also accounts for much observed gene expression heterogeneity in melanoma tumours.

This heterogeneity and reversibility of transcription programs were also shown in vivo using a xenograft mouse model. We also investigated the motive forces behind differential TGF-ß

[121] http://e-collection.ethbib.ethz.ch/eserv/eth:30488/eth-30488-01.pdf

signalling. Smad activation was present in all melanoma cultures irrespective of the presence of a TGF-ß signature, which suggested Smad-independent TGF-ß signalling. The TGF-ß Smad-dependent pathway has long been considered as being central to TGF-ß signalling but it is now recognized that TGF-ß signals via crosstalk with alternative pathways. We investigated alternative pathways but could identify no link between the activation status of several MAPK pathways and the TGF-ß signature. TGF-ß is a multifunctional cytokine which controls aspects of cell proliferation, differentiation, migration, apoptosis, adhesion, angiogenesis, immune surveillance, and survival. TGF-ß was initially defined as a transforming cytokine but it is now understood that TGF-ß has dual roles both as tumor suppressor and tumor promoter.

*To better understand the regulation behind the expression of these opposite behaviors, we studied TGF-ß's cytostatic effect, which plays an important role in its tumor suppressing function and which is lost as melanoma cells become more invasive and metastatic. We identified the Id2 gene as differentially regulated by TGF-ß and link the loss of its regulation to acquired resistance to TGF-ß in invasive phenotype cells. We show that TGF-ß induces cell cycle arrest through induction of*pI5fnk4b *and repression of Id2. Furthermore, Id2 overexpression in proliferative phenotype cells counteracts* pI5fnk4b *induction and consequently protects melanoma cells from TGF-ß-mediated inhibition of proliferation.*

Treating tumours comprised of cells with variably expressing transcription signatures presents a difficult challenge. This is because specific therapies have targeted factors we identify here as being subject to repeated changes in regulation. It is therefore of primary importance we recognize that the existing paradigm for melanoma progression is insufficient for the design of effective therapies.

8.3.4 Pathway Summaries

An excellent summary is also in DeVita, in the chapter by Fisher and Kwong. We first summarize this in the following Table using the materials in the Fisher and Kwong chapter:

Gene	Action
AKT	The AKT gene family consists of AKT1, AKT2, and AKT3, with phospho-AKT as the read out of their overall activation status. Elevated phospho-AKT level was reported to be adversely associated with patient survival.32 More recently, copy number gains of the AKT3 locus were detected in melanomas, suggesting that the AKT signaling point itself may be oncogenic. Interestingly, targeted depletion of AKT3 could trigger apoptosis,33 while AKT1 behaved as a tumor suppressor in melanoma cell lines,34 pointing to poorly understood distinct and overlapping functions of these related family members.
APAF-1	Allelic loss at 12q23 was exhibited by 10 of 24 (42%) melanomas in a study,16 with the common area of loss focused on the APAF-1 locus. LOH correlated tightly with a reduction in Apaf-1 protein levels, as judged by immunohistochemistry. Although no mutations were detected, the loss of expression was determined to be due to silencing in a methylation-dependent manner.
ARF	Reciprocal to the *INK4A*-specific human mutations, *ARF*-specific insertions, deletions, and splice donor mutations have been described in human melanomas (reviewed in Chin et al.14). However, in these cases, either the maintenance of INK4A function or true ARF inactivation was not shown, making it ambiguous whether the genetic disruption of ARF alone is sufficient for tumorigenesis.
BRAF	Somatic activating BRAF mutations are found at high frequency in human melanoma, dominated by a single species of point mutation (T →A nucleotide change), resulting in a valine to glutamate amino acid substitution (V600E). Although the T→A transversion is not classically associated with UV-induced damage, BRAF mutations appear to be more common in melanomas arising on sites with intermittent exposure to UV.23,24 However, melanomas from chronically sun-damaged skin are typically wild type for *BRAF*,

Gene	Action
CDK4A	*CDK4* is a direct target of inhibition by p16INK4A (Fig. 48.1.2) and is a primary regulator of RB activation. If INK4A acts mainly through the RB pathway, it would be predicted that activating CDK4 mutations could functionally substitute for INK4A deletions. Indeed, rare germline mutations of *CDK4* that render the protein insensitive to inhibition by INK4A have been identified in melanoma-prone kindred.8 Somatically, these tumors retain wild-type INK4A function, suggesting that INK4A is epistatic to CDK4 and that Rb pathway deregulation is central to melanomagenesis.
CDKN2A	Its importance is explained in part by its unusual organization, which allows for two separate transcripts and corresponding tumor suppressor gene products to be produced: p16INK4A and p19ARF (Fig. 48.1.2). Loss of p16INK4A results in the suppression of retinoblastoma (RB) activity via increased activation of the CDK4/6-cyclin D1 complex; loss of ARF (p14ARF in human and p19ARF in mouse) results in the suppression of p53 activity through increased activation of MDM2. Thus, deletion of the entire locus accomplishes the inactivation of two critical tumor suppressor pathways: RB and p53.
c-MET	The c-MET gene product and its ligand hepatocyte growth factor/scatter factor (HGF/SF) are known to activate the MAPK pathway, but have many additional functions. It has long been documented in the literature that overexpression of c-MET and HGF is correlated with melanoma progression, with nonfocal amplification of the c-MET locus at 7q33-qter being associated with invasive and metastatic cancers in humans46 and their high levels of expression in murine melanoma cell lines being similarly correlated with metastatic ability in explants.
EGFR	Epidermal growth factor receptor (EGFR) is involved in a complex regulatory loop with the MAPK pathway, where there appears to be bidirectional signaling between EGFR and the RAS kinases. In melanomas, copy number gain of chromosome 7 is linked with overexpression of EGFR, despite the lack of focal amplifications.42 Functionally, although *in vitro* activation of EGFR does not affect melanoma growth, it increased the number of visceral metastases when implanted in severe combined immunodeficiency (SCID) mice.43 Confirmation of EGFR-MAPK cross-talk in melanoma was demonstrated in the inducible H-RAS-driven mouse model,22 where transcriptomic analysis revealed the up-regulation of EGF family ligands including amphiregulin and epiregulin.
INK4A	Human intragenic mutations of *INK4A* that do not affect the *ARF* coding region sensitize germline carriers to the development of melanomas.6 These aberrations can affect the coding region (e.g., exon 1α), either of the 5′ or 3′ untranslated regions (UTRs), the promoter, or splice donor/acceptor sites (reviewed in Sharpless7). This sufficiency of p16INK4A loss for the initiation of melanoma demonstrates that loss of the entire CDKN2A locus is not necessary. In a mouse model engineered to be deficient only for Ink4a (with intact ARF), melanomas formation was observed in cooperation with an oncogenic initiating event (e.g., activated H-RAS), albeit with a longer latency than in mice with deletions affecting the entire locus.
MAPK	The MAP kinase (MAPK) pathway contains some of the earliest elucidated human oncogenes, and subsequent analysis of their mechanisms of action unearthed a prevalence of activating mutations across a wide spectrum of tumor types. The focal point of MAPK activation is the ERK1/2 kinases, which classically mediate the transcription of genes involved in cell proliferation and survival (Fig. 48.1.3), but which have also been shown to regulate differentiation and senescence. In addition, the RAS family of proteins has been shown to feed into the PI3K pathway.
MITF	MITF is a gene critical to the survival of normal melanocytes, and identification of MITF as a central modifier of melanoma created a novel class of oncogenes (along with androgen receptor) termed " lineage addiction" oncogenes.50 That is, a tumor may "hijack" extant lineage survival mechanisms in the presence of selective pressures to ensure its own propagation.
P53	The p53 pathway is critical in maintaining a cell with a normal genome via a multiplicity of mechanisms, including cell cycle checkpoints, DNA damage repair activation, and the appropriate induction of apoptosis. Its centrality in tumor suppression is evidenced by the high rate of its inactivation in solid tumors, with mutations in the TP53 gene well established to be present in over 50% of all tumors. By contrast, the TP53 locus is rarely mutated in human melanomas (reviewed in Chin12), although loss of p53 in mice does cooperate with activated H-Ras to induce melanomas.13 Similar to the LOH at *Cdkn2a* in mice heterozygous for Ink4a/Arf knockout, mutant Tp53 heterozygotes also lose the wild-type allele somatically in H-RAS-driven melanomas. Thus, while p53 itself is spared in human melanomas, inactivation of its pathway is likely to be critical.
PTEN	Of the PI3K pathway mutations that do occur, loss of chromosome 10q encompassing PTEN tumor suppressor is the most frequent, the caveat being that there is likely additional tumor suppressor(s) resident in this region (see below). PTEN normally effects the down-regulation of phosphorylated AKT via suppression of levels of the second messenger PIP3 (Fig. 48.1.3). In various genetically engineered mice bearing solid tumors, PT EN loss can be analogous to p53 inactivation, in that one or the other can provide the " last straw" of oncogenesis. In melanoma, somatic point mutations and homozygous deletions of PTEN are rare. Although allelic loss of PTEN is observed only in about 20% of melanoma, loss of expression of PTEN is reported to be in the range of 40% of melanoma tumors.
RAS	Increasing evidence shows that the three different members of the RAS family are not functionally redundant, with separable roles not only among different tissue types, but even within the same tissue. Reflecting this is the differential mutation and genomic amplification rates of the RAS family members within melanomas: N-RAS is the most frequently targeted (33% of primary and 26% of metastatic melanoma samples17), followed by H-RAS (mainly in Spitz nevi).18 Despite its high incidence in other cancer types, K-RAS is rarely observed in melanocytic lesions.19 Interestingly, although N-RAS mutations are found in 54% of congenital nevi, they are rare in dysplastic nevi,20 implying a distinct evolutionary path from dysplastic nevi to melanoma.

Gene	Action
RB	RB pathway is responsible for preventing cells from incorrectly entering into the cell cycle, and germline heterozygous loss of the *RB1* gene in humans results in the formation of retinoblastoma. The tumor modulating properties of the RB pathway are well established in many solid cancers, and its deregulation in melanoma is no exception, with demonstrable human mutations in *INK4A*, *CDK4*, or *RB1*.
RB1	Finally, germline mutations in RB1 itself have been found to predispose to melanoma in patients who have survived bilateral retinoblastoma.11 These melanomas show a somatic LOH of the remaining wild-type RB1 allele, strongly implying that an intact RB pathway was selected against in the preneoplastic melanocytes. In such patients, the estimates of increased lifetime risk of melanoma range from 4- to 80-fold.
TGF-β	TGF-β family members are active at various stages of human tumors, but its role in melanoma has only recently begun to become clarified. Studies in zebrafish embryos have translated into human data on the role of Nodal in melanomas. Secreted Nodal was shown to be the molecule responsible for zebrafish axial duplications when human melanoma cells were implanted into the embryos. Subsequent immunohistochemical analysis of Nodal in human melanocytes and melanomas showed a significant correlation with tumor progression. Knockdown of Nodal in metastatic cell lines reduced their invasive capacity *in vi tro* and their growth in mouse xenografts.
WNT	WNT signaling has long been implicated in a wide variety of cancers including breast and colorectal. Its activation of downstream transcriptional events has been hypothesized to control lineage commitment and differentiation fates as well as self-renewal properties. Indeed, the WNT pathway has been linked to major developmental decisions in neural crest derivatives, with a differentiation bias toward the melanocytic lineage.

And in comparison from Bennett we have the following summary which relates specifically to melanoma:

Gene	Location	Change	Melanomas % (no of samples) tested)	Data Source (cultured or not)	Also earlier in progression?
APAF1	12q23	Methylation	42 (24)	Uncultured	No. Advanced only
APC	5q21-22	Methylation (+1 mutation)	16 (94)	Both	Not known
BRAF	7q34	Activating mutation	47 (2805)	Both	51% of benign nevi
CDKN2A(p16, ARF)	9p21	Deletion Methylation Mutation (p16) Total	50 (119) 19 (59) 9 (760) 78	Uncultured(higher in cultured)	Known only for mutations: 0% in nevi, 12% in dysplastic nevi
CDKN2B(p15)	9p21	Deletion	36 (74)	Cultured	Not known
CTNNB1(b-catenin)	3p22	Activating mutation	6 (408)	Both	Not known
KIT	4q12	Amplification ± activating mutation	14 (36)	Uncultured	Not known
MITF	3p14	Amplification	10 (119)	Cultured	Not known
MYC	8q24	Amplification	1–40	Uncultured	Not nevi. Most in advanced
NRAS	1p13	Activating mutation	21 (2517)	Both	21% of benign nevi
PTEN	10q23	Mutation Deletion Total	17 (501) 13 (119) 28	Both Cultured	Not known
PTPRD	9p23	Deletion	6 (119)	Cultured	Not known
RB1	13q14	Mutation	6 (67)	Both	Not known
STK11(LKB)	19p13	Mutation	10 (144)	Both	Not known
TBX2	17q23	Amplification	43 (46)	Cultured	Not known
TP53(p53)	17p13	Mutation	9 (232)	Uncultured	Not known

8.3.5 Pathways and Methylation[122]

The changes in pathways control elements, by loss of an element of by the hyper-activation of one, frequently is related to methylation of cytosine. Methylation is the replacement of a H atom with a CH_3 methyl group on position 5 of cytosine. How does this happen? Also what does it result in? The result is changes in pathway elements and the above discussions relates many of them. The gene is changed and then the pathway disturbed. The result can be a cancer.

How this happens will discuss shortly.

The issue of having a methylation and then change in DNA during transcription has been discussed by Esteller who states:

The best-known epigenetic marker is DNA methylation. The initial finding of global hypomethylation of DNA in human tumors5 was soon followed by the identification of hypermethylated tumor-suppressor genes, and then, more recently, the discovery of inactivation of microRNA (miRNA) genes by DNA methylation. These and other demonstrations of how epigenetic changes can modify gene expression have led to human epigenome projects14 and epigenetic therapies.5 Moreover, we now know that DNA methylation occurs in a complex chromatin network and is influenced by the modifications in histone structure that are commonly disrupted in cancer cells... DNA methylation has critical roles in the control of gene activity and the architecture of the nucleus of the cell. In humans, DNA methylation occurs in cytosines that precede guanines; these are called dinucleotide CpGs. CpG sites are not randomly distributed in the genome; instead, there are CpG-rich regions known as CpG islands, which span the 5′ end of the regulatory region of many genes. These islands are usually not methylated in normal cells. The methylation of particular subgroups of promoter CpG islands can, however, be detected in normal tissues.

Esteller continues:

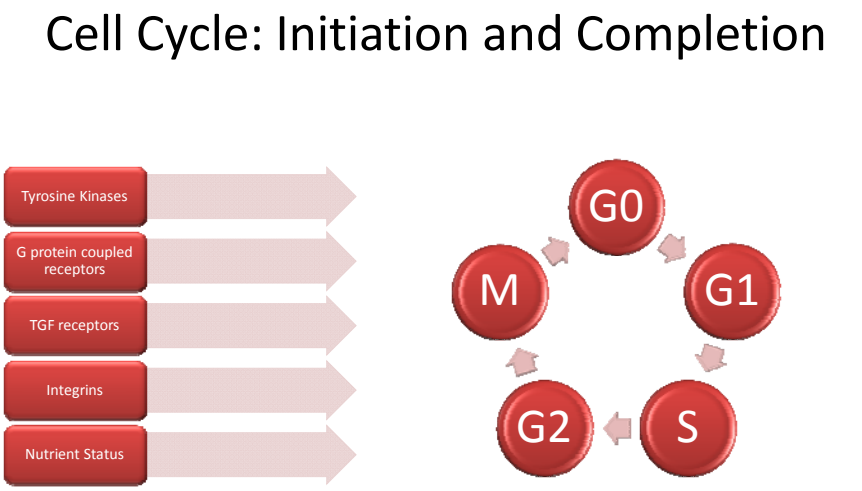
Normally, certain testis specific genes, genes that encode melanoma antigens, or specific proliferation-linked genes38 are silent in somatic cells because promoter-region CpG islands are methylated. In some cancer cells, by contrast, these promoter regions undergo demethylation, and the usually repressed genes become expressed. Two notable examples of the hypomethylation mechanism are the activation of PAX2 (a gene that encodes a transcription factor involved in proliferation and other important activities of cells) and the activation of the let-7a-3 miRNA gene, which has been implicated in endometrial and colon cancer.46,47 The hypomethylation of DNA can have unpredictable effects.

8.3.6 Melanoma Generation

The question then is what causes the loss of control as we have seen in the previous section. There are two possible routes. First the route of changes that let the pathways start to diverge

[122] See the papers by Miranda, Zilberman, Jacobsen, Robertson, Strathdee for a current description of the methylation process and cancer.

from homeostasis and the second, most likely resulting in the first, where a change occur in the genetic elements.



One of the key issues then is the deregulation of pathways resulting in loss of control of the normal homeostatic state. As Weinberg states[123]:

"Evidence of an even more cunning strategy for destabilizing this control circuit has been found in the genomes of a small number of both sporadic and familial melanomas. In these cancers, point mutations in the CDK4 gene, the R24C mutation, create CDK4 molecules that are no longer susceptible to inhibition by the family of INK4 molecules (p15, p16, p18, and p19). … CDK needs to be mutated in order for a cancer cell to derive proliferative benefit."

As Bennett states:

Overall, we now know of huge numbers of molecular changes that have been described in advanced melanomas compared with melanocytes. Some commoner and well-known changes are, for example,

- *activating mutations of BRAF …*
- *silencing of E-cadherin expression …, and*
- *acquisition of telomerase activity …*

[123] Weinberg, Cancer, p. 298.

But hundreds more, mostly unfamiliar, are emerging from comparative gene expression profiling of melanomas from various stages …

What can we make of all these changes? Can we determine which are central to malignancy itself and thus are candidate therapeutic targets? Certainly, we still have much to learn, but as a start, we can seek to distinguish primary from secondary changes.

A primary event in progression would be a cellular change that is clonally inherited, that contributes to the eventual malignancy, and that occurs independently rather than as a secondary result of some other oncogenic change.

These events are either

(i) genetic (gene mutation, deletion, amplification or translocation), or

(ii) epigenetic (a heritable change other than in the DNA sequence, generally transcriptional modulation by DNA methylation and ／ or by chromatin alterations such as histone modification).

In clonal evolution of cancer, such a primary event would initiate a new, more progressed, clone with a growth advantage over its neighbors, or an alternative selective advantage such as migration …

8.3.7 Some Simple Calculations

We now want to perform a simple calculation. Let us assume methylation is a cause, that is the breaking of a CH bond on cytosine position 5. The question we pose is what causes that and what chance do we have.

We summarize an initial calculation in the following Table. In this Table our objective is to measure the ratio of the x-ray photon energy to the CH bond energy to assure ourselves that we have more than enough energy to break a bond. The details below clearly demonstrate that fact.

CH Bond	99.3	Kcal/mole
Avogadro Number	6.00E+23	
Conversion	2.85	Kcal/mole
Conversion	0.1239	eV/molecule
CH Bond	4.32	eV/molecule
CH Bond width		A
Wavelength of x-ray photon	1.5	A
Wavelength of x-ray photon	1.50E-08	m
Frequency of x-ray photon	2.00E+16	Hz
Energy of x-ray photon	1.33E-17	E=hf Joule
Speed of Light	300,000,000	m/sec
Planck's Constant	6.63E-34	Joule/sec
Conversion	11900	Joule/mole
Conversion	0.1239	eV/molecule
	7.43E+22	Joule/ev
Energy CH bond	5.81E-23	Joule
Energy of x-ray photon	1.33E-17	E=hf Joule
Ratio x-ray Energy to Bond Energy	2.28E+05	

This tells us that the ratio of x-ray energy to CH bond energy is over 100,000 and that the x-ray has an excellent chance to knock off the H and have it replaced by a methyl group.

Calculating probabilities are much more complex and have not been attempted here. The issue here is that we have a flux of x-ray photons and then we have DNA going through many transcription processes. Thus we seek to determine the chance that:

1. An x-ray photon gets to a location to break bonds

2. That the location is also at the time the transcription is occurring.

With regard to total exposures as currently practiced, we have from the paper by Brenner the following Table:

Study Type	*Relevant Organ*	*Relevant Dosage (mGy or mSv)*
Dental radiography	Brain	0.005
PA Chest radiography	Lung	0.01
Lateral Chest Radiography	Lung	0.15
Screening Mammogram	Breast	3
Adult CT	Stomach	10
Barium Enema	Colon	15
Neonatal CT	Stomach	20

Recall that a 100 rem equals 1 Gy. Thus a neonatal CT would be the equivalent of 2,000 rems.

9 CANCER MODELS FOR UNDERSTANDING, PREDICTION, AND CONTROL

We will now consider what are the essential elements and tools for modeling cancers. The first step is to re-establish the goals of a model and then its structure. Finally we will lead into the interrelationship between a model and the data which is used to justify it.

Many authors have developed models concerning pathways and also cancer. The books by Klipp et al and that of Szlassi et al are excellent overviews of the area with significant detail. The Klipp et al book is a truly superb discussion regarding pathways and modeling alternatives. The books by Bellomo et al and Wang are directed specifically at cancer modeling but unfortunately they lack adequate pathway dynamics to be of substantial use. Yet they are the only books available within the focused area.

At the core, we want a model which reflects the following qualities:

1. Based Upon Reality: The model must at its core be based upon the known reality. It must conform to what we currently know and understand. Namely it must reflect in its core the elements which we consider critical and the temporal and spatial dynamics of those elements. The model must be based upon a tempero-spatial system of measurable quantities; linked in some kinetic manner using reasonably well understood processes.

2. Predictability: Any modeling must, if it is to have any credibility, have the ability to predict, to say what will happen, and then to have that prediction validated. Although the ability may be statistical in nature the statistical confidence must be justifiable. We know all too well that many things are correlated, yet not causal, and not predictable.

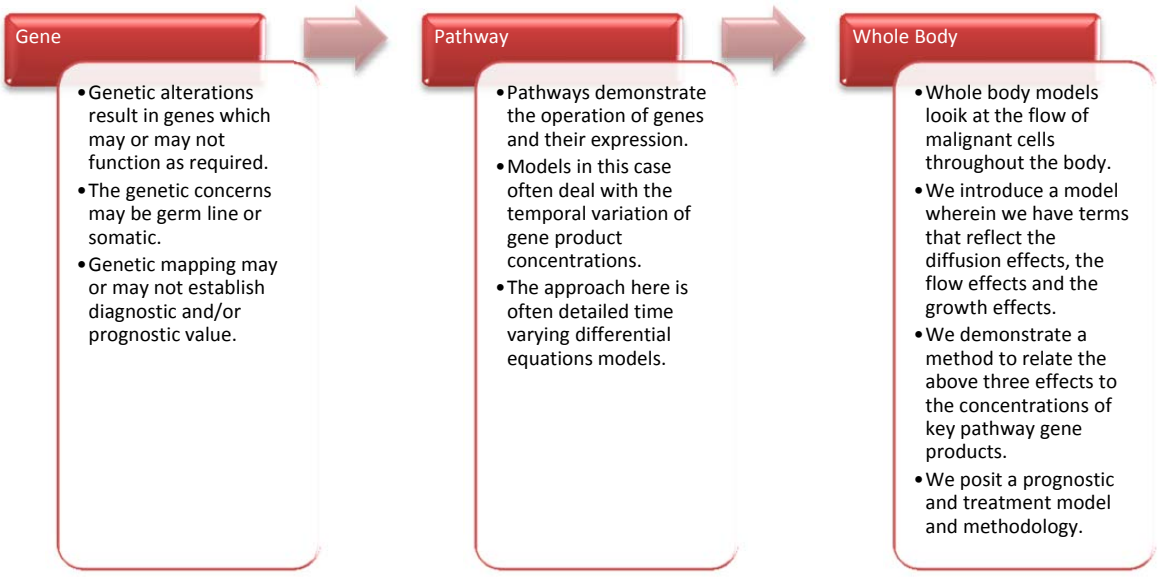
3. Measurable: One must be able to measure and then predict the quantities which make up the model. Many of the modeling systems include proteins but they react in some zero-one format. We know in reality that we have concentrations, or better yet specific numbers of proteins, produced in a cell. Yet we cannot yet measure the number of each of these proteins. We all too often can at best measure their presence or absence. However, is it not the case that it is the excess or the low density of some set of proteins which shift reactions, and that reactions are often concentration dependent.

4. Modellable: We want a system which can be modeled. It must reflect the measurable quantities in space and time and the tempero-spatial dynamics of them, using techniques that we can then use for prediction and validation.

In this paper we examine and analyze several models of cancer. Specifically we look at intracellular, extracellular and full body models. We attempt to establish a linkage between all of them. Many researchers have looked at the gene level, the pathway level and the gross flow of cancer cell level, namely whole body. Connecting them has been complex to say the least.

But herein we look at the pathway level and a whole body level and demonstrate the nexus, physically, and from this we argue that one can construct both prognostic tools as well as methodologies to deal with metastasis.

The following graphic lays out the flow of development and its implications as we detail them herein.



9.1 CHARACTERISTICS OF CANCER

What are the key characteristics of cancer? As Weinberg notes in the figure below, normal cells have the following functions:

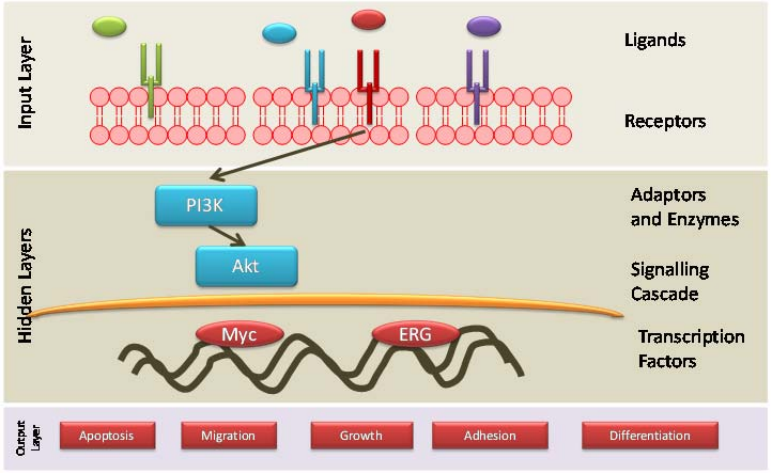
1. Growth: Growth is often distinguished from cell multiplication, or mitosis. Growth is cell expansion, the production of new proteins, the expansion of intra-cellular fluids, and the inclusion of exogenous factors found in normal cell life. In a sense cells consume and cells can get fat! The cells produce and absorb. They consume so they can both grow and duplicate, via simple mitosis.

2. Migration: Cells often have an ability to find their place and stay there. Blood cells flow out into the vascular system, and they tend to stay there until their natural course is complete. Basal cells remain basal as they progress up through the epithelium. Melanocytes remain melanocytes and remain in the basal layers, when benign.

3. Apoptosis: Cells die. They are programmed to do so and they do so in a clean and elegant fashion leaving no mess behind. This is in contrast to cell death with a diseased or damaged cell in which case the immune system must take over and remove the debris.

4. Differentiation: Cells have the ability to differentiate and perform specific functions.

5. Adhesion: Cells stick to adjacent cells, keeping their place in a collection of many other differentiated cells, and performing specific cellular functions.



The following represent several of the characteristics found in cancer cells.

1. Genetic Alterations: The genes in cancer cells are often markedly changed. We have demonstrated that the literature has for melanoma, and for many other cancers, laid out various genetic profiles, some connected with pathway alterations and others just recognized.

2. Cell Cycle Activation: Cells enter mitosis when activated generally y external growth stimuli and in turn activate growth pathways. It is possible that as a result of certain genetic pathway alterations that they may get activated endogenously, rather than exogenously. In either case we have activated the cell cycle and if not controlled the cell will complete mitosis and a doubling will occur. Unlimited cell cycle activation will result in the unlimited growth of cancer cells and the displacement of normal cells and the functions that those cells provide.

3. Loss of Normal Apoptosis: Loss of normal cell death is a major factor of cancer cells; they just do not die off.

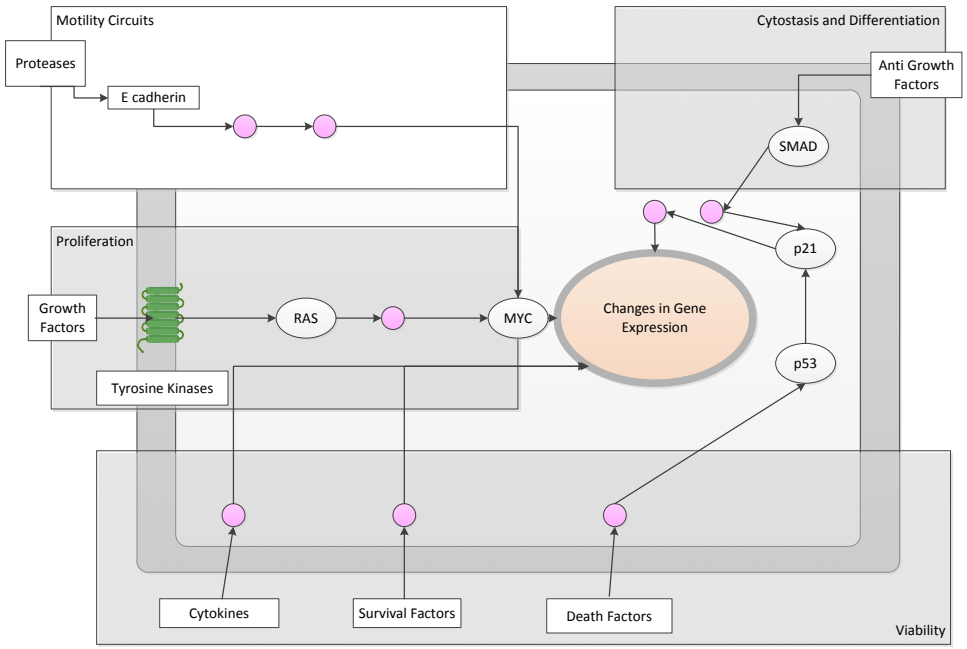
4. Loss of Spatial Stability: The spatial instability is also a known attribute. The melanocyte for example just starts to wander, and then to replicate, and fails to die off through apoptosis.

5. Loss of Specific Functionality: Cancer cells lose all functionality. If a glandular cell, it no longer is. As a malignant melanocyte it no longer produces melanosomes for the skin.

6. Development of Alternative Metabolic Capabilities: This is in effect the Warburg Effect. Namely the metabolism of the cancer cell is anaerobic rather than oxidative.

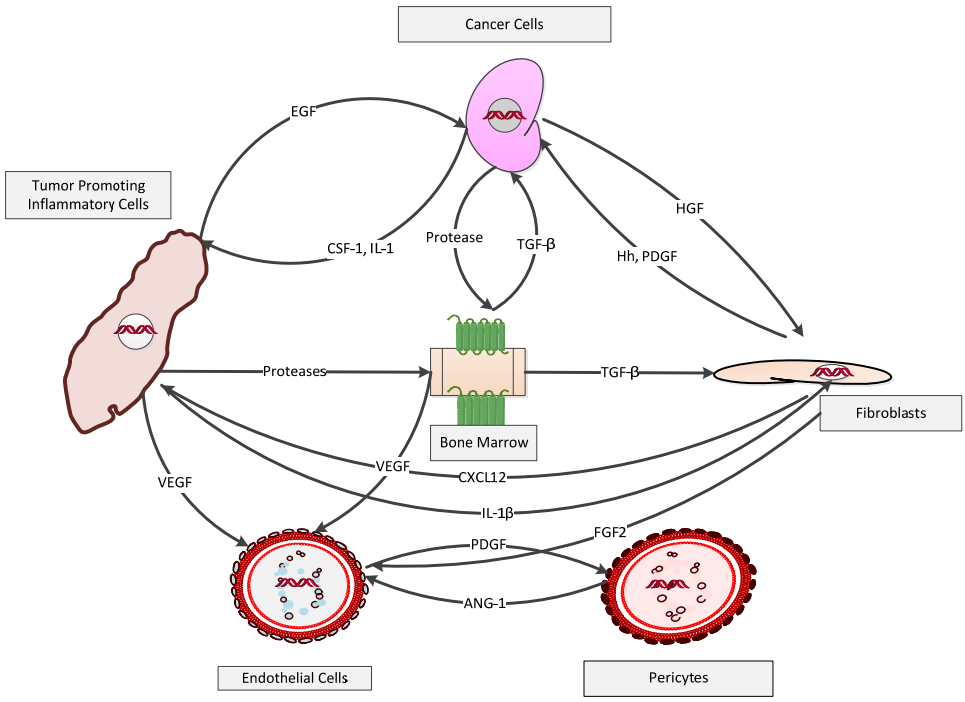
7. Establishment of a Stem Cell Motif: We have discussed this at length before but it is simply the hypothesis that a small collection of cells control the metastatic process and that this small collection is a clone which may at this point be mutating independently. This implies that the remaining cancer like cells are; (i) not capable of separate metastatic, and (ii) if one removes the

CSC then one halts the cancer and the remaining cells will go through some form of apoptosis. Also, (iii) the identification of the CSC is separate and apart from all other cells.



9.2 CANCER: AN EVOLVING PUZZLE

Cancer is an evolving puzzle. What we present here is not expected to be definitive. The challenge is that as we learn more about the cell and about cells in environments, it will most likely be reflected in our understanding of cancer cell dynamics. For example, below we have a modification from Weinberg showing the interaction between cancer cells and other cells in the body.



Several recent papers have been published on the details of cancer genetics which make the understanding a continuous process of complexity. Let me first provide a brief précis of how we have progressed to this point:

1. The clone. It has been asserted that almost all cancers begin with a single aberrant cell, the clonal source. From this one cell we have generate everything else. One single cell then replicates in an uncontrolled manner.

2. The Vogelstein Paradigm: The Vogelstein Paradigm (VP) states that the clone is created in some predictable sequence of gene changes and that these changes can be detected and perhaps blocked.

3. The genetic profile: This concept uses the wealth, also excess, of gene mutation data available from microarray analysis to determine "profiles" for various cancers attempting to gain prognostic information as well as "individual" profiling for treatment. In many ways the micro array tool provides "too much data", akin to the comment in Amadeus when the Emperor was asked about Mozart's music, and he remarked "too many notes". Namely the wealth of data is essential but the ability of the human processor is not quite up to it yet.

4. The pathway model: In this case we use pathways as a means to understand what is going wrong in a cell by cell basis. Then we try to block aberrant pathways to have the tumor no longer function as it has to that point. We have argued that this approach has a strong core, namely a model which can be verified and improved, but at the same time it lacks two major factors; (i) is does not deal with intercellular communications well enough, (ii) it does not deal with the issues of what causes the loss of gene activity and homeostasis well enough.

Now there have been several papers in NEJM discussing results on several cancers, kidney and AML, acute myeloid leukemia. Combined they tell and interesting tale. I have already commented on the kidney paper by Gerlinger et al but will add to it in this analysis.

As Gerlinger et al state:

Multiregion genetic analysis of four consecutive tumors provided evidence of intratumor heterogeneity in every tumor, with spatially separated heterogeneous somatic mutations and chromosomal imbalances leading to phenotypic intratumor diversity (activating mutation in MTOR*) and uniformity (loss-of-function mutation in* SETD2 *and* PTEN*).*

Of all somatic mutations found on multiregion sequencing, 63 to 69% were heterogeneous and thus not detectable in every sequenced region. Heterogeneous patterns of allelic imbalance were found in all tumors, and ploidy heterogeneity was found in two tumors. Therefore, we found that a single tumor-biopsy specimen reveals a minority of genetic aberrations (including mutations, allelic imbalance, and ploidy) that are present in an entire tumor.

Thus with this study we see significant genetic variability. The sequencing of genetic changes and the expectation of clonal consistency seems to be at variance.

In contrast, to justify the clonal progression, as Walter et al state regarding AML:

A unique aspect of the biology of leukemia is that hematopoietic cells freely mix and recirculate between the peripheral blood and the bone marrow. Clones that persist and grow over time must retain the capacity for self-renewal. Mutations in new clones must confer a growth advantage for them to successfully compete with ancestral clones. The result is that these secondary-AML samples are not monoclonal but are instead a mosaic of several genomes with unique sets of mutations; this mosaic is shaped by the acquisition of serial mutations and clonal diversification. Similarly, recent analysis of de novo AML samples with the use of whole-genome sequencing showed that relapse after chemotherapy is associated with clonal evolution and acquisition of new mutations.

Analysis of individual cancer cells may reveal additional layers of genetic complexity. Recent studies of B-cell acute lymphoblastic leukemia have shown that serial acquisition of cytogenetic abnormalities in that disease most often occurs through a branching hierarchy and only rarely follows a simple linear path…. Our study has several clinical implications.

First, the distinction between the myelodysplastic syndromes and secondary AML currently relies on manual enumeration of bone marrow myeloblasts, a standard that is subject to interobserver bias but nonetheless drives major decisions about treatment for patients with small differences in myeloblast counts. Ultimately, identifying the patterns of pathogenic mutations and their clonality in bone marrow samples from patients with myelodysplastic syndromes should lead to greater diagnostic certainty and improved prognostic algorithms.

Neither studies presented intracellular pathways models which could be verified as state machines leading to malignant processes nor did they provide any basis for the genetic variations

observed. These two factors will be essential in a better understanding of these diseases. However we see strong hematopoietic clonality and non-hematopoietic non-clonality.

The question one may ask is: do the cancer cells as they progress in a metastatic manner do so in a random ever changing manner unconnected from one another or is there some rational underlying physical basis for the changes in a manner in which the cancer has become an alter-organism in the human host? Is cancer a "slime mold" atop the human?

9.3 WHAT DO WE MODEL IN CANCER?

The key question we ask is just what is it we are modeling in cancer cell dynamics. Let us consider some options:

First: This type of model focuses on the genes, and their behavior. It is basically one where we examine the gene type and its product.

Second: This type of model falls in several subclasses. All begin with protein pathways and the "dynamics" of such pathways. But we have two major subclasses; protein measures and temporal measures. By the former we mean that we can look at the proteins as being on or off, there or not there, or at the other extreme looking at the total number of proteins of a specific type generated and present at a specific time. By the latter, namely the temporal state, we can look at the proteins in some static sense, namely there or not there at some average snapshot instance, or we can look at the details over time, the detailed dynamics. In all cases we look at the intracellular dynamics only.

Let us consider the two approaches.

i. On-Off: In this approach the intracellular relationships are depicted as activators or inhibitors, namely if present they allow or block an element in a pathway. PTEN is a typical example, if present it blocks Akt, if absent it allows Akt to proceed and enter mitosis. p53 is another example for if present we have apoptosis and if absent we fail to have apoptosis. These are simplistic views. This is a highly simplistic view but it does align with the understanding available say with limited microarray techniques. This is an example of the data collection defining what the model is or should be.

ii. Density: This is a more complex model and it does reflect what we would see as reality. The underlying assumptions here are:

a. Genes are continually producing proteins via transcription and translation.

b. Transcription and translation are affected at most by proteins from other genes acting as repressors or activators. There are no other elements affecting the process of transcription and translation. Not that this precludes any miRNA, methylation, or other secondary factors. We shall consider them later. In fact they may often be the controlling factors.

c. The kinetics of protein production can be determined. Namely we know the rate at which transcription and translation occur in a normal cell or even in a variant. That is we know that the production rate of proteins can be given by:

$$\frac{\partial n_j(t)}{\partial t} = \sum_{k=1}^{K} \lambda_k n_k(t)$$

Here we have production rates dependent on the concentration of other proteins. The processes related to consumption are not totally understood (see Martinez-Vincente et al). We understand cell growth, as distinct from mitotic duplication, but the growth of a cell is merely the expansion of what was already in the cell when at the end of its mitotic creation. In contrast, we understand apoptosis, the total destruction of the cell, we also understand that certain proteins flow outside the cell or may be used as cell surface receptors, but the consumption of these is not fully understood. Yet we can postulate:

$$\frac{\partial n_j(t)}{\partial t} = -\sum_{k=1}^{K} \mu_k n_k(t)$$

This is based upon the work of Martinez-Vincente et al which states:

All intracellular proteins undergo continuous synthesis and degradation (Mortimore et al., 1989; Schimke, 1970). This constant protein turnover, among other functions, helps reduce, to a minimum, the time a particular protein is exposed to the hazardous cellular environment, and consequently, the probability of being damaged or altered. At a first sight, this constant renewal of cellular components before they lose functionality may appear a tremendous waste of cellular resources.

However, it is well justified considering the detrimental consequences that the accumulation of damaged intracellular components has on cell function and survival (Goldberg, 2003). Furthermore, protein degradation rather than mere destruction is indeed a recycling process, as the constituent amino acids of the degraded protein are reutilized for the synthesis of new proteins (Mortimore et al., 1989; Schimke, 1970).

The rates at which different proteins are synthesized and degraded inside cells are different and can change in response to different stimuli or under different conditions. This balance between protein synthesis and degradation also allows cells to rapidly modify intracellular levels of proteins to adapt to changes in the extracellular environment. Proper protein degradation is also essential for cell survival under conditions resulting in extensive cellular damage. In fact, activation of the intracellular proteolytic systems occurs frequently as part of the cellular response to stress (recently reviewed in Cuervo, 2004b; Goldberg, 2003). In this role as 'quality control' systems, the proteolytic systems are assisted by molecular chaperones, which ultimately determine the fate of the damaged/unfolded protein (Fig. 1).

Damaged proteins are first recognized by molecular chaperones, which facilitate protein refolding/repairing. If the damage is too extensive, or under conditions unfavorable for protein repair, damaged proteins are targeted for degradation. Protein degradation is also essential

during major cellular remodeling (i.e. embryogenesis, morphogenesis, cell differentiation), and as a defensive mechanism against harmful agents and pathogens (recently reviewed in Cuervo, 2004a; Klionsky, 2005).

We have also discussed this process with regards to the function of ubiquitin, which marks proteins for elimination. As Goldberg states:

Proteins within cells are continually being degraded to amino acids and replaced by newly synthesized proteins. This process is highly selective and precisely regulated[1], and individual proteins are destroyed at widely different rates, with half-lives ranging from several minutes to many days. In eukaryotic cells, most proteins destined for degradation are labelled first by ubiquitin in an energy requiring process and then digested to small peptides by the large proteolytic complex, the 26S proteasome.

Indicative of the complexity and importance of this system is the large number of gene products (perhaps a thousand) that function in the degradation of different proteins in mammalian cells. In the past decade, there has been an explosion of interest in the ubiquitin–proteasome pathway, due largely to the general recognition of its importance in the regulation of cell division, gene expression and other key processes[1]. However, the cell's degradative machinery must have evolved initially to serve a more fundamental homeostatic function — to serve as a quality-control system that rapidly eliminates misfolded or damaged proteins whose accumulation would interfere with normal cell function and viability.

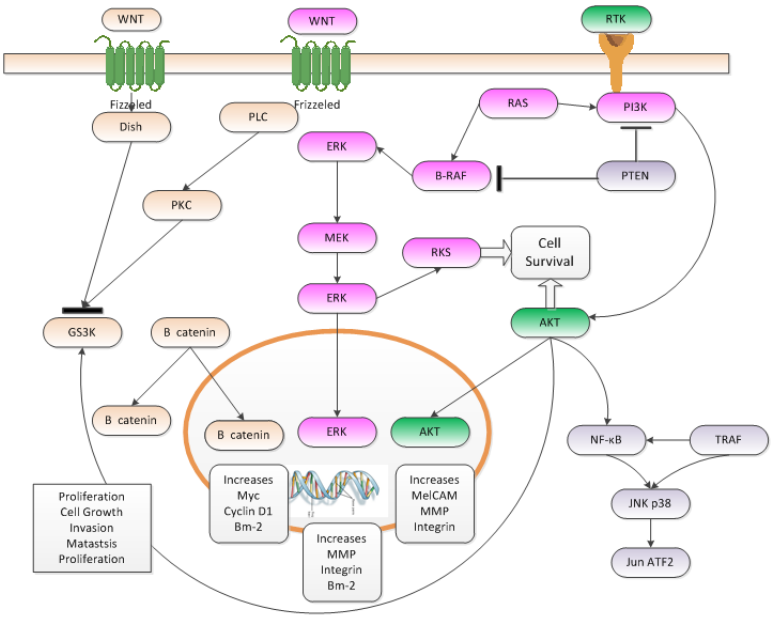
Also we refer to the recent review work of Ciechanover which details the evolution of this understanding.

In contrast the proteins are consumed and thus the negative sign. In toto we have:

$$\frac{\partial n_j(t)}{\partial t} = \sum_{k=1}^{K} (\lambda_k - \mu_k) n_k(t)$$

as a total balance of proteins. This assumes we have a production mechanism for each of the proteins, namely their genes and the activators and repressors as required.

d. Pathway Dynamics must be meaningful. Let us consider the pathway as shown below. This is a typical melanoma pathway we have shown before.



Now let us consider PTEN blocking BRAF and Akt. Now physically it is one molecule of PTEN needed for each molecule of BRAF and PI3K. But what if we have the following:

$$n_{PTEN}(t) < n_{BRAF}(t)$$
$$n_{PTEN}(t) > n_{PI3K}(t)$$

Here we have PTEN blocking some but not all the BRAF and PTEN blocking all the PI3K. At least at time t. Do we have an internal mechanism which then produces even more PTEN? One must see here that we are looking at the actual numbers of PTEN, real numbers reflecting the production and destruction rates. We know for example that if we have a mutated BRAF then no matter how much PTEN we have an unregulated pathway.

Now it is also important to note that this "model" and approach is distinct in ways from classic kinetics, since the classic model assume a large volume and concentrations in determining kinetic reaction rates of catalytic processes. Here we assume a protein binds one on one with another protein to facilitate a pathway.

Thus knowing the dynamics of individual proteins, and knowing the pathways of the proteins, namely the temporary adhesion of a protein, we can determine several factors:

1. The number of free proteins by type
2. The pathways activated or blocked
3. The resultant cellular dynamics based on activated pathways.

It should be noted that we see pathways being turned on and off as we produce and destroy proteins. There is a dynamic process ongoing and it all depends on what would be a stasis level of proteins by type. The question is; are cells in stasis or are they in a continual mode of regaining a temporary stasis?

This also begs the question, that if as we have argued, that cancer is a loss of stasis due to pathway malfunction, then can this be a process of instability in the course of a normal cell? Namely is there in the dynamics of cell protein counts, unstable oscillator type modes resulting in uncontrolled mitotic behavior. Namely can a cell get locked into an unstable state and start reproducing itself in that state, namely an otherwise normal cell.

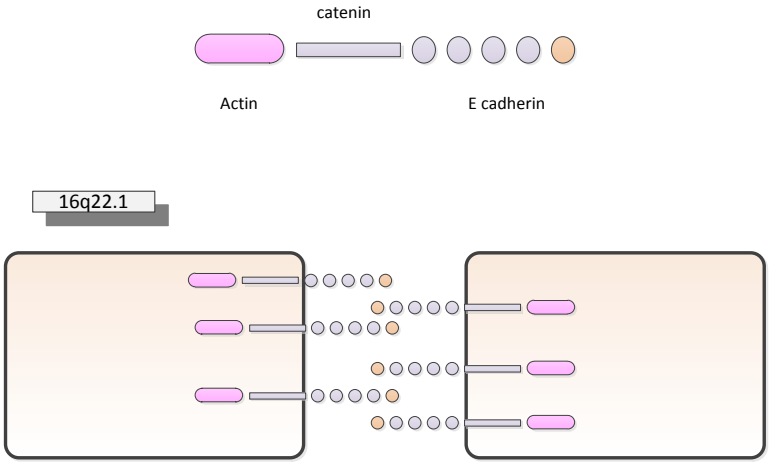
e. Total intracellular dynamics can be modeled yet the underlying processes are still not understood and the required measurements are yet to be determined.

9.3.1 Intercellular Dynamics:

Here we look at the intercellular dynamics as well, not just as a stand-alone model. By this methodology we look at intercellular communications by ligand binding and the resulting activation of the intracellular pathways. We must consider both the intercellular signalling between like cells but also between unlike, such a white cells perhaps as growth factor inhibitors and the like. We also then must consider the spatiodynamics, namely the "movement" of the cells, or in effect the lack of fixedness or specificity of function. This becomes a quite complex problem.

There are two functions we examine here:

a. Intercellular binding or adhesion: E cadherin is one example that we see in melanocytes. Pathway breakdown may result in the malfunctioning of E cadherin.



The above demonstrated E cadherin in melanocyte-keratinocyte localization. The bonds are strong and this stabilizes the melanocyte in the basal layer. If however the E cadherin is compromised then the bond is broken, or materially weakened, and the melanocyte starts to wander. Movement for example above the bottom of the basal layer and upwards is pathognomonic of melanoma in situ. Wandering downward to the dermis becomes a melanoma. Thus the pathways activating E cadherin production is one pathway essential in the inter-cellular dynamics.

b. Ligand production and receptor production: Here we have cells producing ligands, proteins which venture out of the cell and become signalling elements in the intercellular world. We have the receptor production as well, where we have on the surface of cells, various receptors, also composed of cell generated proteins, which allow for binding sites of the ligands and result in pathway activation of some type. For example various Growth Factors, GF proteins, find their way to receptors, which in turn activate the pathways. Wnt is an example of one of these ligands which we have shown above.

It can also be argued that as ligands are produced and as the "flow" throughout the intercellular matrix, we can obtain effects similar to those in the Turing tessellation models. Namely a single ligand may be present everywhere but density of ligands may vary in a somewhat complex but determinable manner, namely is a wavelike fashion.

Thus, as with intracellular proteins, we have with intercellular ligands the following:

$$q(x,t) = E\left[Number\ of\ ligand\ Proteins; x, t\right]$$

and we would have some dynamic process as:

$$\frac{\partial q(x,t)}{\partial t} = R(x,t)\frac{\partial^2 q(x,t)}{\partial x^2} + \ldots$$

This is akin to the Turing model used in patterning of plants and animals. Namely the concentration of a ligand, and in turn its effect, may be controlled by

9.3.2 Total Cellular Dynamics:

In this case we would want a model which reflects the total body spatiotemporal dynamics This type of models is an ideal which may or may not be achievable. In a simple sense it is akin to diffusion dynamics, viewing the cancer cells as one type of particle and the remaining body cells as another type. The cancer cells have intercellular characteristics specific to cancer and the body cells have functionally specific characteristics. Thus we could ask questions regarding the "diffusion" of cancer cells from a local point to distant points based upon the media in between. The "rate" of such diffusion could be dependent upon the local cells and their ability for example to nourish the cancer cells as well. In this model we could define an average concentration of cancer cells at some position x and time t as:

$$p(x,t) = E\left[Number\ of\ Cancer\ Cells; x, t\right]$$

and we would have some dynamic process as:

$$\frac{\partial p(x,t)}{\partial t} = D(x,t)\frac{\partial^2 p(x,t)}{\partial x^2} + \ldots$$

This is a diffusion like equation and is a whole body equation. Perhaps knowing what the rate of diffusion is on a cell by cell basis may allow one to determine the most likely diffusion path for the malignancy, and in turn direct treatment as well.

This is of course pure speculation since there has been to my knowledge any study in this area. Except one could imagine a system akin to PET scans and the like which would use as input the surface markers from a malignancy and then the body diffusion rates to plot out in space and time the most likely flow of malignant cells and thus plan out treatment strategies. Although this model is speculative we shall return again to it in a final review of such models since it does present a powerful alternative.

This concept of total cellular dynamics is in contradistinction to the intercellular transport. In the total cellular dynamics model we regard the model as one considering the flow of altered cells across an existing body of stable differentiated cells.

We may then ask, what factors drive cancer cells to what locations? One may putatively state that cancer cells will follow the path of least resistance and/or will proceed along "flow lines" consistent with what propagation dynamics they may be influenced by.

We shall examine these intercellular factors in a later chapter.

9.4 INTRACELLULAR DYNAMICS: AN EXAMPLE

We now consider in more formalistic terms how to develop models. There are three approaches under consideration:

Boolean Networks: The model assumes discrete time on or off nets controlled by the dynamics of the pathway. The Boolean model consists of two elements: first, an assumption that a gene or protein is on or off, second, that the control of that constituent is a Boolean control consisting of AND, OR and NOT Boolean type functions dependent upon precursor constituents. We discuss this in some detail related to PTEN. The Boolean approach is interesting in that it works well at times identifying non-dynamic pathways using massive micro-array data.

Bayesian Networks: The Bayesian model assumes that the value may be analog not binary and that there exists a graph which is what is the desired output. It uses a Bayes model which yields the best fitting graph given data from a microarray and assuming that there are linkages consisting of probabilities in a directed manner. The Bayesian may become time varying as well. As with the Boolean approach it works well with ascertaining linkages and graphs using microarray data and in this case the microarray data may be of an analog nature not just binary.

State Models: These are continuous time and continuous state models of specific pathway concentrations where we employ enzymatic reaction rate models that we have discussed previously. We have used these models in other circumstances and they have proven their worth. Also these models are often more amenable to stochastic effects as well as examination for observability and controllability.

9.4.1 Boolean Networks

The simplest model for pathways is the Boolean model. This has been described in Klipp et al in simple terms. A classification of Boolean Networks has been done in Gershenson. It is based upon three simple assumptions.

First, genes and their products are either on or off, expressed or not expressed. This denies concentration effects.

Thus as an example we may have PTEN and Akt, and they are either 1 or 0. Thus we have the four states, {PTEN, Akt} as 00, 10, 01, 11. Not all may exist, we will show that next.

Second, the effects on a set of gene products on other gene products can be expressed by a Boolean expression.

Thus, for example we have the Binary or Boolean expression:

Akt = NOT PTEN.

Thus if PTEN is 0 then Akt is 1 and likewise if PTEN is 1 then Akt is 0. The states 11 and 00 are not allowed.

Third, this is a discrete time state system where we have the kth instance influencing the k+1 th state.

Thus as above we should state:

Akt(k+1)=NOT PTEN(k)

We may add asynchrony and probabilistic transitions as well to the model.

Now let us formalize the model a bit. We assume that there are N genes or gene products that are of interest. Then the state of the system at any time is:

$$x(k) = \begin{bmatrix} x_1(k) \\ \dots \\ x_N(k) \end{bmatrix}$$

where the xs can take on only 0 or 1 in value, on or off.

Now we assume that for each x(k) we have some Boolean equation of the following type:

$$x_j(k+1) = (x_1(k)ANDx_3(k))NOTx_4(k)ORx_6(k)$$

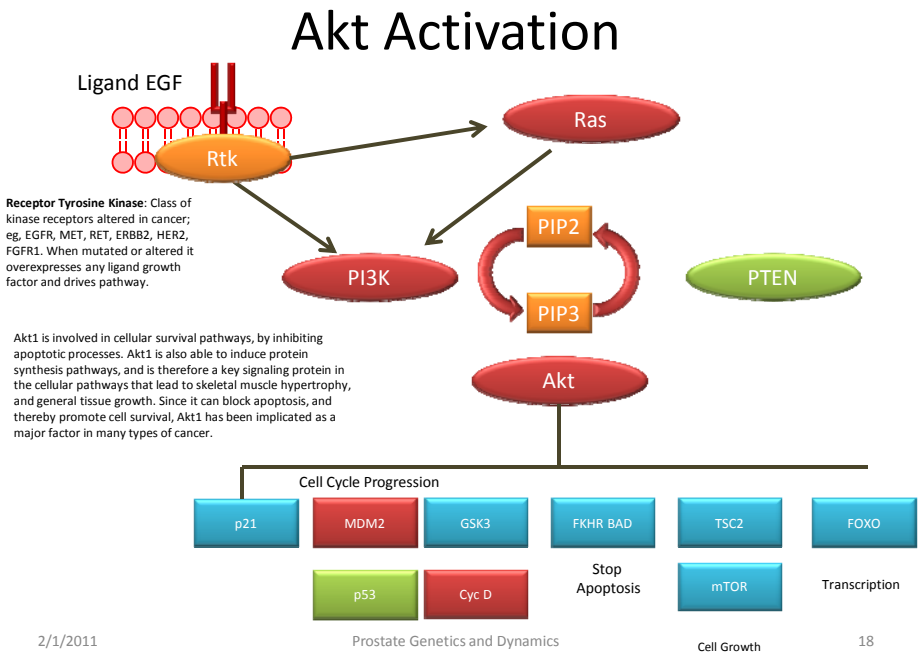
We now ask where did this equation come from. We defer that for the moment. But let us assume that we can find that for every one of the x values. The we have:

$$x(k+1) = F(k+1,k)$$

where

$$F(k+1,k) = \begin{bmatrix} f_1(k+1,k; x_1(k)...x_N(k)) \\ ... \\ f_N(k+1,k; x_1(k)...x_N(k)) \end{bmatrix}$$

Now let us return to our cancer model and look at a simplified set of it as shown below. First, from Lin et al we have for the simple model showing that "*PTEN Decreases AR Protein Levels via Promotion of AR Degradation"* which we show below;



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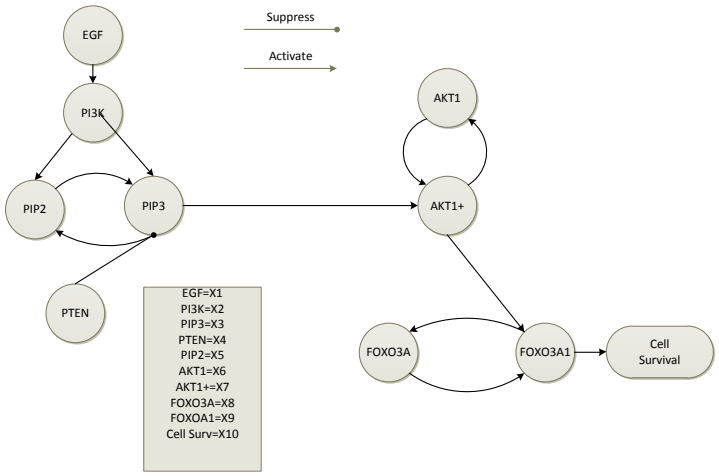
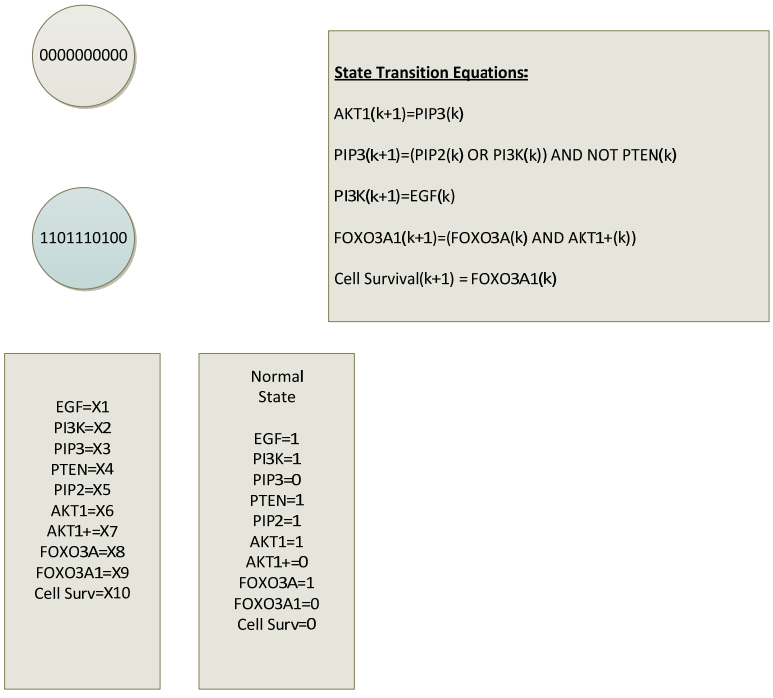
Then from the above we can generate a simple model of pathways with some detail as below:



Now in this model we assume that an EGF ligand drives PI3K which moves PIP3 to activate AKT to AKT+1 the activated form which drives FOXO to enhance cell survival. We see 10 states all of which are binary. Ideally we would have 2^{10} possible states, roughly a million. But the state transitions will prohibit this.

The following depict the transition equations, the Boolean f functions from above, as we have somewhat arbitrarily created them, and the stable state.



Note that we can assume that EGF is one and PTEN is 1 and thus PTEN blocks everything. Note also that only 5 state equations are specified. The others are just stable states. We can now take

any states, namely ones where EGF and PTEN go from 0 to 1 or 1 to 0 and see what happens. Note that we have in this model the assumption that PI3K needs EGF. Also if PTEN is on then it suppresses cell growth via FOXO.

One can use microarray technology to validate this model. However it has significant drawbacks.

First it is binary. Real world models are not that way.

Second it is discrete time, again not a reflection of reality.

Third, it assumes binary reactions, and the real reactions are a bit more subtle.

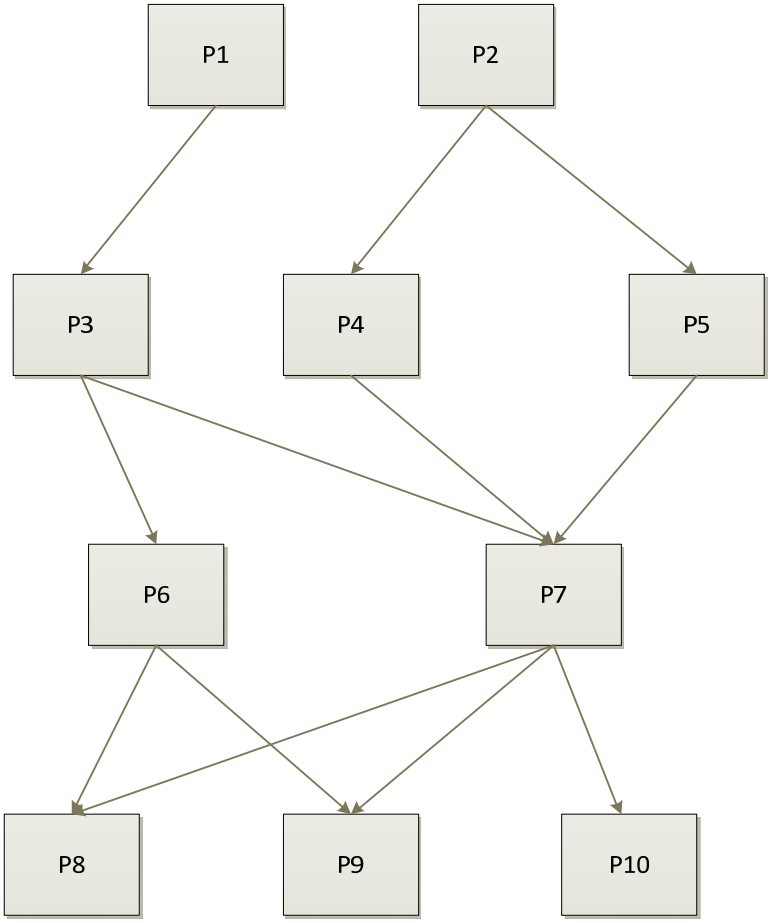
The use of the pathway data is oftentimes effective at the gross level (see Kim et al). Also the ability of correlating pathway models with microarray data is also of significant value. Oftentimes the microarray data itself is binary and thus it maps fairly well onto this model (see Driscoll and Gardner).

Expansion of this model to non-discrete times and random transitions has been shown in the wok of Shmulevich et al (three papers in 2002, 2002, 2002)

9.4.2 Bayesian Networks

In the development of a Boolean Network we assumed two things; first that the proteins or other relative constituent was either on or off and second that the interaction between constituents was controlled via some Boolean function of AND, OR, NOT type combinations. Now in the Bayesian world we assume Bayes type relationships and then try to infer dependency thereupon.

Let us begin by looking at the following network. We assume that the Ps are influencing the Ps they are connected to in some manner. For simplicity we shall assume they are all positive influences.



Now in a Bayes world we generally have some data and we then try to infer from the data the structure as we see it above.

Let us look at the above flow. Here we have assumed a priori that there are well defined influencing factors. But what if all we had was 10 products and we just thought that they somehow interact. What we want to do is use some logical method to arrive at the chart above. How could we approach this?

Let us assume we have some microarray data. Let us further assume that it is simple microarray data showing genes on or off.

We could write in general the following (see Theodoridis and Koutroumbas, pp 64-68):

$$p(P10, P9, P8, \ldots P1) = p(P10 \mid P9, \ldots P1)\, p(P9 \mid P8, \ldots P1) \ldots p(P2 \mid P1)\, p(P1)$$

Now that does not reflect the graph, G, which we had shown at the top. One may look at the set of all possible graphs, say:

$$\Gamma = \{G_i\}$$

where we have any possible G as a set. We are really interested in the "best" G give say a data set D.

So let us go to the data set, D. What can we use to assist in determining the best possible G. Let us assume we have a microarray. The microarray presents data in one of two forms; active or no effect. Namely the array elements lights up if active and does not light up is not active. Let us assume we have 20 samples and we test for the ten products. We get an array as below. Here red is active.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
P1																				
P2																				
P3																				
P4																				
P5																				
P6																				
P7																				
P8																				
P9																				
P10																				

From this we have a data set given by;

$$d_i = \begin{bmatrix} d_{i,1} \\ \dots \\ d_{i,10} \end{bmatrix}$$

and

$$D = \{d_1, \dots d_N : N = 1, \dots, 20\}$$

We can now look at the problem in a Bayesian sense. Let G be the graph and D the data as we demonstrated above. Now as a Bayes approach we can seek:

$$max\left[log\, P\left[G|D \right] \right] =$$

$$max\left[log\, P\left[D|G \right] + log\, P\left[G \right] + c \right]$$

We thus seek to maximize the Bayes score. We seek the G which given the D gives the best result.

Now we can model the system as follows:

$$P_i = \sum_{k=1,\neq i}^{10} a_{i,k} P_k$$

and

$$d_i = b_i P_i$$

The a values are determined by the graph G. We can add noise to the measurement and even to the system. We can then find the set of a that given the D set minimize the measure we stated above. Since we can assume that we have:

$$P_i = \sum_{k=1,\neq i}^{10} a_{i,k} P_k + n_i$$

and

$$d_i = b_i P_i + w_i$$

where they are zero mean Gaussian, then the metric used to attain a minimum on the data set D to determine the graph G is simple, yet a complex calculation.

Namely we have (see Shmulevich and Dougherty):

$$max\left[log\, P\left[G \middle| D \right] \right] =$$

$$max\left[log\, P\left[D \middle| G \right] + log\, P\left[G \right] + c \right]$$

or

$$max\left[-\sum_{k=1}^{10} \sum_{i=1}^{20} \left(d_{i,k} - \hat{d}_{i,k} \right)^2 \right] + log\, P\left[G \right]$$

where we have used the estimated d for the specific graph G being tested. We do this for every possible graph, G. That is a very computationally complex calculation. Indeed it can become non-calculateable.

We demonstrate in the next section that we can measure analog values for the measurements as well as for the desired connections.

9.4.3 Microarrays

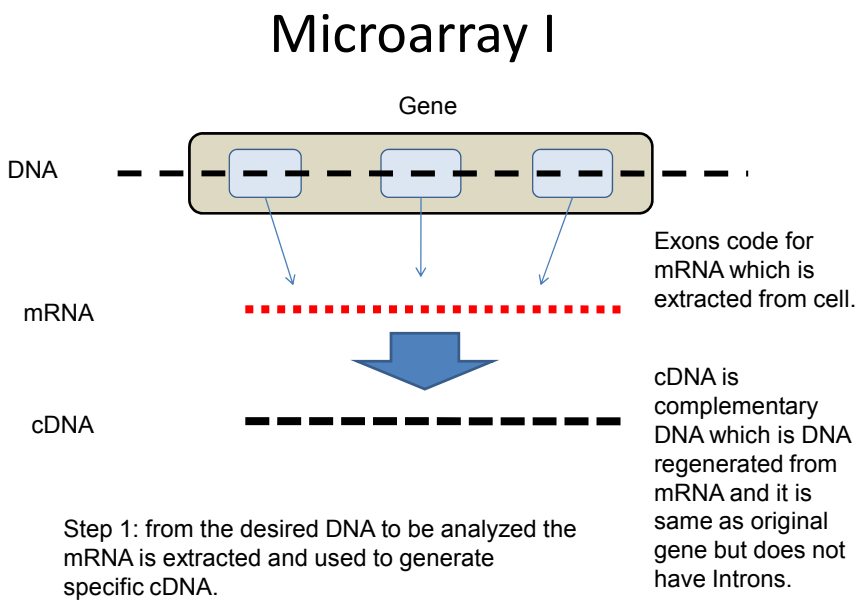
We briefly look at microarrays in some detail since we will use them in the Bayesian case as well in the full system modeling case. This is but one of the many methods available to develop identification tools to determine such things as reaction coefficients.

Microarrays are simply a set of matrices where we take samples from many cells and place them in an N by M matrix, say using rows for genes and columns for cell samples. By selecting the rows such that we can place in each row a gene specific binding site, which we can even tag with

some visible marker, we can then determine if on a sample by sample basis we have genes being expressed, namely that they bind at the binding site. It is also possible to attempt to ascertain the concentration of the gene or gene product by having the amount of binding in each sample be reflected by a linear or similar color change. We can say have red for no binding, yellow for some and green for a great deal, or whatever color combination we so desire. Indeed it may then be possible to calibrate for relative concentrations in each cell entry via a colorimetric measurement.

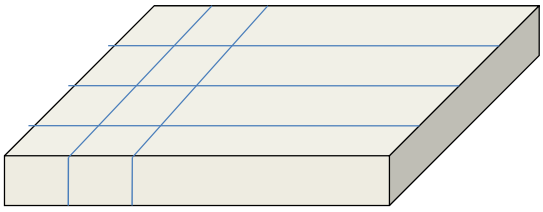
Microarrays is a unique approach which allows for the analysis of millions of samples, it is a marriage of high tech solid state chip technology with DNA bonding. We describe it in the following four steps, each step accompanied by a Figure.

Step 1: The first step in a micro array is the production of cDNA, or complementary DNA. cDNA is that set of nucleotides which account for the encoding of mRNA. It does not include the non-coding regions which are the introns.



Step 2: In a separate environment we make the microcell. This is created in a manner identical to the making of integrated circuits which entails photo-masking techniques. Instead of silicon we used nucleotides. The array has millions of small holes in an array like manner. Each hole we fill with nucleotide, one nucleotide at a time.

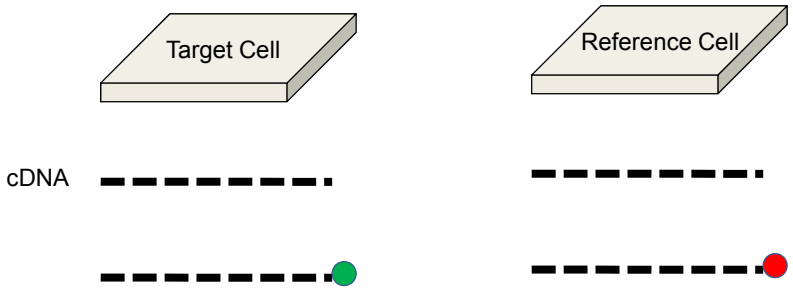
Microarray II



Step 2: Using photolithographic techniques, nucleotides for selected cDNA segments are built up cell by cell creating a collection of binding sites of single stranded DNA sections about 25 nucleotides deep/long on the surface of an NXM array. Each cell becomes sticky for a specific DNA segment.

Step 3: Now we take two DNA samples, one from what we call the Target, the plane we wish to categorize. We then take the segments we collected in step one and tag then with green or red tags, green say for the Target and Red for the Reference.

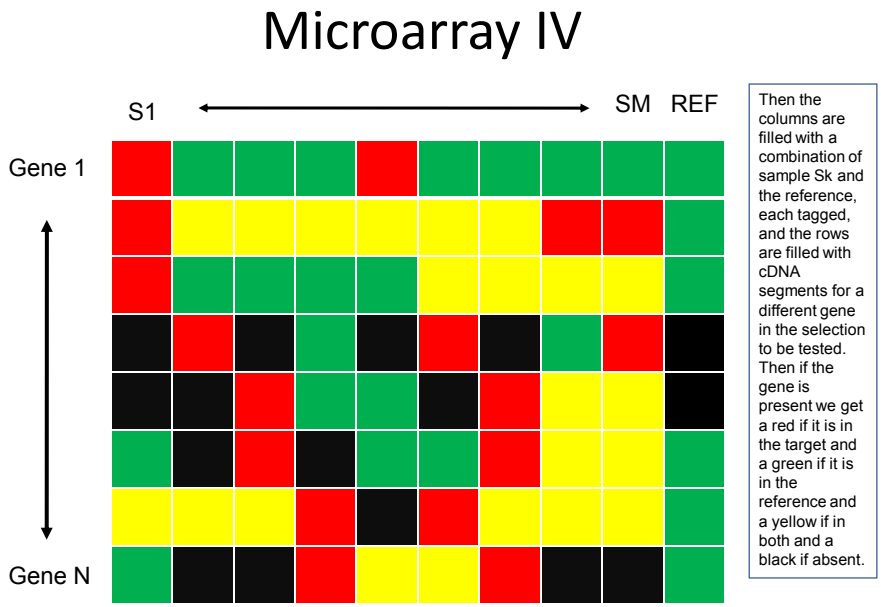
Microarray III



Step 3: For the DNA to be analyzed and a “Reference” target DNA, the mRNA is extracted from each and the cDNA is produced for every gene in the cells to be analyzed, and then it is tagged with a dye which is red for one and green for the other. Typically we tag the target red and Reference green.

Step 4: We then take the samples from the differing plants, one in each column, and look at the array. If the microarray cell has the gene sequence we are seeking to march, and the Target has

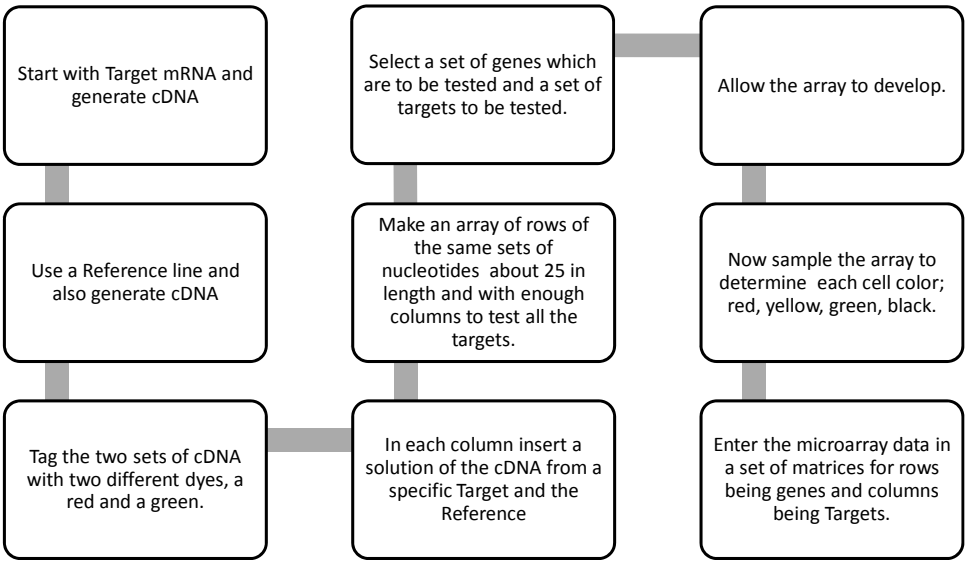
that sequence, it will bond and stick. If the Reference has it, it too will bond. If we just get the Target the cell will be green, if we just get the Reference the cell is red, if we get both the cell turns yellow, and if we have neither the cell is black. The result of a sample scan is shown below.



Now, we even get to try and look at the intensity of the red, green, or yellow. This we can try to see how much is expressed not just whether it is or is not. We will not discuss that here. In the above matrix we can see that many genes are expressed in one or both or none. If we have enough genes than we can argue we have the basis for an exceptionally good means to develop a classification.

In the following Figure we summarize the microarray process.

Microarray Summary



This is a brief summary of microarray technology. We refer the reader to the volume of literature available and referenced to herein.

9.4.4 Bayesian Network Summary

The Bayesian network approach can be posed as a general problem. Yet it is often delimited by assuming a priori that certain graphs, G, and no possible, and the minimization or maximization process then is performed over the subset of graphs. There is often still quite a great deal of calculation required.

On the negative side the Bayesian approach as is the case for the Boolean approach does not take into account the underlying chemical dynamics that we have been observing. In addition for most of the pathways we are looking at we already know the key pathway structure and it is the reaction kinetics that we are looking for.

Thus Boolean and Bayesian are useful for identifying pathways whereas the full system model works when the pathway is somewhat well understood and we are looking more for the dynamics and details of the reactions. In a sense they are useful at different stages of the process.

9.4.5 State Dynamic Networks

The state dynamic network model for pathways is the heart of what we have been developing herein. It consists of the following:

1. Intracellular Pathways: The description of pathways within the cell and a description which focuses on concentrations of products and exogenously extracellular products that affect the pathways.

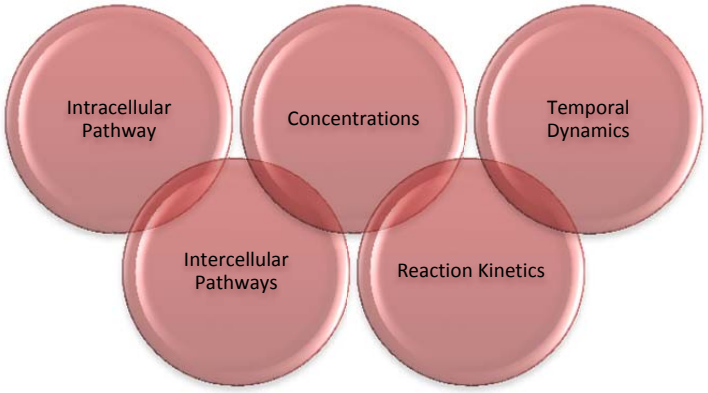
2. Intercellular Pathways: This is the modeling of pathways between and amongst the cells. It is intercellular signalling pathways where receptors and their associated ligands are studied. Again this may generally be focused on concentrations of ligands and similar extracellular and intercellular communicators.

3. Concentrations: The focus is on concentrations of the gene products. The issue here is that it is concentrations which reflect reality and although binding and pathway control may be seen as promoted or inhibited by other proteins, the reality of reaction kinetics is that it is concentrations which are reflective not just single molecule presence or absence. This element is dramatically different from what we see in Boolean or Bayesian models. Here with the modeling with concentrations we are modeling closer to reality.

4. Reactions: Reaction kinetics describes how one substance can control the conversion of another substance into a third. The reaction kinetics are essential to the system dynamics model. The challenge is to know and understand which of the reaction models apply. For example are there rate limiting factors due to concentration limitations of the reaction. However as we have shown before, we are at so low a concentration in a cell that we may very well be dealing with protein to protein bonding, not looking at concentration based reactions. We have not examined this in detail but it will be considered later.

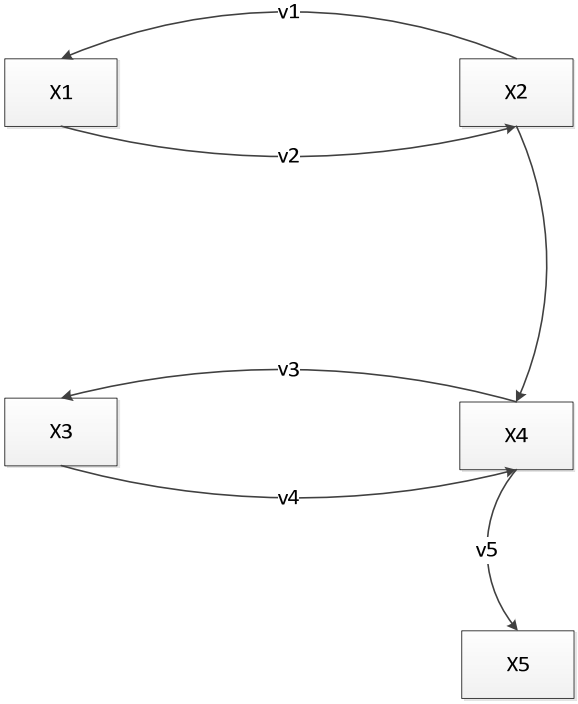
5. Time Variations: The models are all time varying. Thus understanding reaction kinetics from a temporal perspective is essential. This may take our knowledge to the limit and perhaps beyond at the current time.

We graphically demonstrate these factors below:



In order to best understand the system dynamic model we again briefly return to the reaction rate model and its part in defining the reaction rate equations.

Let us consider a simple model as below:



Recall that if we have a pathway with say N=5 constituents then we have the following formula where the rates v are yet to be determined:

$$\frac{d[x_1(t)]}{dt} = v_1 - v_2$$
$$\frac{d[x_2(t)]}{dt} = v_2 - v_1$$
$$\frac{d[x_3(t)]}{dt} = v_4 - v_3$$
$$\frac{d[x_4(t)]}{dt} = v_3 - v_4$$
$$\frac{d[x_5(t)]}{dt} = v_5$$

Now let us return to the rate analysis. We will use the paper by Segel and Slemrod as the basis. This paper presents an excellent analysis of the quasi steady state assumptions for rate and reaction with enzymatic issues as we will encounter.

Assume we have the following reaction:

$$E + S \rightleftarrows C \rightarrow E + P$$

We further assume that the forward rate from E+S to C us k_1 and the reverse is k_{-1} and the forward rate from S to E+P is k_2. Then we have the following:

$$\frac{dE}{dt} = -k_1 ES + k_{-1} C + k_2 C$$
$$\frac{dS}{dt} = -k_1 ES + k_{-1} C$$
$$\frac{dC}{dt} = k_1 ES - k_{-1} C - k_2 C$$
$$\frac{dP}{dt} = k_2 C$$

Note that as we have discussed before the equations are nonlinear due to the product terms. We could linearize them by assuming that we are dealing with small changes or we can use the quasi steady state assumptions which reduces the equations yet keeps the nonlinearity. We shall do the later. Also note the mapping of the above specific reactions to the generalized equations we have used above. Also note that when we have two constituents using an enzyme we see the enzyme as a single combine entity and the initial components as two separate ones. Also we have reaction rates thus dependent to the product of the concentrations on each side of the reaction.

Now let us proceed with the enzymatic reaction. We can restrict the analysis to the following conditions:

$$E(0) = E_0$$
$$S(0) = S_0$$
$$C(0) = 0$$
$$P(0) = 0$$

This is a simple defined initial state when we have just the initial entities which we desire to react. But we also can readily show:

$$E(t) + C(t) = E_0$$

Thus we can simplify the result to what we have below by elimination:

$$\frac{dS}{dt} = -k_1 (E_0 - C0S + k_{-1} C$$
$$\frac{dC}{dt} = k_1 (E_0 - C)S - k_{-1} C$$
$$S(0) = S_0$$
$$C(0) = 0$$

These are still rather complex differential equations and we will now assume that the rates of each are dramatically different. Namely one proceeds quite quickly and the other slowly. That being the case we can thus assume that one is steady state and the other dominates the observable time variation. This is in essence the quasi steady state assumption. This assumption is:

$$\frac{dC}{dt} \approx 0$$

Then we obtain:

$$C = \frac{E_0 S}{K_m + S}$$

where

$$K_m = \frac{k_{-1} + k_2}{k_1}$$

and finally we have the single differential equation for S, which is:

$$\frac{dS}{dt} = \frac{k_2 E_0 S}{K_m + S}$$

This can be applied to the above initial generic form depending upon what the reaction is. Simple reactions do not involve an enzymatic catalyst as we have shown here but in general we will always be dealing with some nonlinearities.

The above deals with four of the five elements we discussed previously: intracellular pathway which was given, rate reactions, concentrations and temporal dynamics.

9.5 IDENTIFICATION, REGRESSION AND ITS DRIVERS

The issue of determining the reaction constants in the system model is of significant importance. In this section we address the issues regarding such estimation, often called system identification. In simplest terms we may have determined the following:

1. Pathway: We can ascertain what the pathway and related constituents are with some certainty.

2. Reaction Models: We may for each set of related elements in the pathways be able to determine what the reaction dynamics can be.

3. Dynamic Model: Having the first two we may then be able to posit a dynamic model for all of the material concentrations of constituents, and yet now have specific values for the reaction rates.

4. Unknown Reactants: There may be unknown reactants or even noise in the system. These may be modeled by an extended identification process or noise. We will demonstrate both herein.

5. Spatial Dynamics: We have referred to the spatial dynamics from time to time but as we have stated, albeit critical, it lacks adequate experimental data to make any progress at this time.

6. Stem Cell Dynamics: We believe that there is substantial evidence of a stem cell model with a CSC in cancer. However, at this time we cannot differentiate these. Yet using the methodology as we develop it, we believe that understanding the dynamics of CSC cancer cells can be determined.

9.5.1 Identification

This section addresses the ability to determine the detailed concentrations of each of the colorants in a cell if one knows the cell effective optical length and the extinction coefficients for each of the constituents. The models for performing these tasks also show what the maximum resolution that can be achieved as well and the maximum number of constituents. The results in the maximum bounding resemble the same results that are found in such areas as ascertaining the accuracy in ambiguity functions for phased arrays. The latter problem was solved by the author in the mid-1970s.

9.5.1.1 Network Model

As we have discussed earlier, the network may be characterized by a matrix differential equation of the form:

$$\frac{dx(t)}{dt} = f(x,t) + w(t)$$

where

$$x = \begin{bmatrix} x_1 \\ \dots \\ x_n \end{bmatrix}$$

and

$$f(x,t) = \begin{bmatrix} f_1(x,t) \\ \dots \\ f_n(x,t) \end{bmatrix}$$

and

$$w = \begin{bmatrix} w_1 \\ \dots \\ w_n \end{bmatrix}$$

We assume that w is a white noise zero mean process and that the f have reasonable mathematical properties. This generalization can be specified for any know gene or gene product network.

We will assume that we can linearize this model. Namely we can do what we had done earlier when looking at the ability to linearize an enzymatic reaction network. This will assume that we have small variations in the constituents. This yields:

$$\frac{dx(t)}{dt} = Ax(t) + w(t)$$

where, as above, x is an n by 1 vector and A is an n by n matrix evaluated about the stability point. That is:

$$A = \begin{bmatrix} \frac{\partial f_1}{\partial x_1} \cdots \frac{\partial f_1}{\partial x_n} \\ \cdots\cdots\cdots\cdots \\ \frac{\partial f_n}{\partial x_1} \cdots \frac{\partial f_n}{\partial x_n} \end{bmatrix}$$

Now the steady state profile would be:

$0 = Ax$
and
x_0
such that
$0 = Ax_0$

where we have defined the steady state concentrations. Arguably if we know the steady state concentrations then A must yield the zero vector resulting therefrom.

9.5.1.2 Measurement Model

Now we use the microarray method to measure. The issue however is to measure amongst groups of common cells. Consider the following microarray form. We have n sample across the columns and m gene or gene related samples across the columns.

The microarray can be constructed so that the color spectrum of the reflected light is a function of the density of the targeted gene or gene counterpart. Thus the microarray must be aligned to deal with samples from large, organized and segmented samples. Below we show 15 gene products and 19 samples. The relative concentrations are also shown.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
G1	1	3	1	2	3	1	2	3	2	1	3	1	1	3	2	2	3	2	1
G2	2	2	2	3	2	2	3	2	3	2	2	2	2	2	3	3	2	3	2
G3	3	2	2	3	1	3	3	1	3	3	2	2	3	1	3	3	2	3	3
G4	3	2	2	3	1	3	3	1	3	3	2	2	3	1	3	3	2	3	3
G5	4	2	4	2	1	4	2	1	2	4	2	4	4	1	2	2	2	2	4
G6	6	1	5	1	2	6	1	2	1	6	1	5	6	2	1	1	1	1	6
G7	3	2	5	2	3	3	2	3	2	3	2	5	3	3	2	2	2	2	3
G8	2	4	5	5	4	2	5	4	5	2	4	5	2	4	5	5	4	5	2
G9	3	5	6	6	5	3	6	5	6	3	5	6	3	5	6	6	5	6	3
G10	1	7	6	6	6	1	6	6	6	1	7	6	1	6	6	3	7	6	1
G11	2	9	2	7	7	2	7	7	7	2	9	2	2	7	7	3	9	7	2
G12	5	7	1	7	4	5	7	4	7	5	7	1	5	4	7	2	7	7	5
G13	6	4	2	8	4	6	8	4	8	6	4	2	6	4	8	2	4	8	6
G14	7	3	3	8	4	7	8	4	8	7	3	3	7	4	8	8	3	8	7
G15	2	2	4	9	3	2	9	3	9	2	2	4	2	3	9	9	2	9	2

Now the color of the cell will reflect the concentration of what the cell has been targeted for, a gene or gene product. Thus by measuring the cell color we can infer the cell concentration and thus the concentration of that specific product in a cell.

The first simplistic identification model will be developed. We thus make the following assumptions:

1. We assume we have n gene products and m samples.

2. We assume we have for each (n,m) tuple a sample which is a color and that we can calibrate color to concentration. We will discuss this in some detail in the next section.

3. We assume that the samples are either from a steady state model or are from a dynamic model and labeled accordingly.

4. We assume that the samples are an (n,1) tuple and we then have k of them.

5. We may also have knowledge of the A matrix whereby we know that certain linkages exist, are positive or negative and that certain linkages do not exist. Thus from Boolean or Bayesian analyses we may have a priori knowledge.

6. We then pose the following problem:

Given :

$$x(i) = \begin{bmatrix} x_1(i) \\ ... \\ x_n(i) \end{bmatrix}$$

$i = 1, M$

Find

$$\hat{A} = \begin{bmatrix} \hat{a}_{1,1} ... \hat{a}_{1,n} \\ ..... \\ \hat{a}_{n,1} ... \hat{a}_{n,n} \end{bmatrix}$$

such that A yields the best fit to the data and subject to the known constraints.

Now what do we mean by best fit to the data? We mean that at steady state the product of any data set and A is Ax=0. But we have k data sets and from these data sets we want to obtain an estimate of A, namely its element entries, subject to whatever constraints we may know a priori.

We know the following:

$Ax = 0$

or

$$\sum_{i=1}^{M} a_{j,i} \tilde{x}_i(k)$$

for j=1,M and all k measurements;

where $\tilde{x}$ is a measured value

Then again the above is subject to whatever a priori constraints we also have. In effect we are looking for a entries which can minimize a metric of the form:

$$min \sum_{k=1}^{m} \left[\sum_{i=1}^{n} a_{j,i} \tilde{x}_i(k) \right]^2 ; \forall j$$

That is for each j entry we want to find the a which minimize the metric above, subject to the constraints. Thus for any j we can say:

$$min \sum_{k=1}^{M} \left[\sum_{i=1}^{n} a_{j,i} \tilde{x}_i(k) \right]^2$$

implies

$$g(a_{j,i}) = \sum_{k=1}^{M} \left[\sum_{i=1}^{n} a_{j,i} \tilde{x}_i(k) \right]^2$$

and

$$\frac{\partial g(a_{j,i}; i = 1,n)}{\partial a_{j,i}} = 0; \forall j$$

Thus simplistically; if we have enough measurements we can estimate the a values by performing the above analysis. Let us look at a simple example.

$$min \sum_{k=1}^{m} \left[a_{j,1} \tilde{x}_1(k) + a_{j,2} \tilde{x}_2(k) + a_{j,3} \tilde{x}_3(k) \right]^2$$

implies

$$g(a_{j,i}) = \sum_{k=1}^{m} \left[a_{j,1} \tilde{x}_1(k) + a_{j,2} \tilde{x}_2(k) + a_{j,3} \tilde{x}_3(k) \right]^2$$

and

$$\frac{\partial g(a_{j,i}; i = 1,n)}{\partial a_{j,i}} = 0, \forall j$$

We can look at the details as follows:

$$g(a) = \sum_{k=1}^{N} \left(a_{11} x_1 + a_{12} x_2 + a_{13} x_3 \right)^2$$

thus

$$\frac{\partial g}{\partial a_{11}} = \sum_{k=1}^{N} 2 a_{11} x_1 \left(a_{11} x_1 + a_{12} x_2 + a_{13} x_3 \right) = 0$$

or

$$\sum_{k=1}^{N} x_1 \left(a_{11} x_1 + a_{12} x_2 + a_{13} x_3 \right) = 0$$

or

$$a_{11} R_{11} + a_{12} R_{12} + a_{13} R_{13} = 0$$

where

$$R_{ij} = \sum_{k=1}^{N} x_i x_j$$

Now we will use a heuristic approach to this solution. It will be a modified Newton method, discussed later. It should be remembered that we have here a simple dynamic system at steady state. We have assumed:

1. Linear relationships for reaction rates.
2. Steady state.
3. An assumed set of initial network maps with some minimal guidance as to the entries, even at the 0,1 level.
4. A wealth of microarray data adequate to assist a convergence.

Now let us assume the following:

Let

$$a_j(k) = \begin{bmatrix} a_{j1} \\ \dots \\ a_{jn} \end{bmatrix}$$

Thus we will develop an estimator for each of these vector elements which comprise the matrix A. The above are the rows of A.

Now we posit as per Newton's method:

$$\hat{a}_j(k+1) = \hat{a}_j(k) + \kappa_j \left[\hat{A}(k)x(k) - \hat{A}(k-1)x(k-1) \right]$$

where

$$\hat{A}(k) = \begin{bmatrix} \hat{a}_1(k) \\ \dots \\ \hat{a}_n(k) \end{bmatrix}$$

we choose the weighting constant on a trial and error basis. This works as follows:

1. We start with an initial set of estimates for the a values. These may be from a Boolean or Bayesian estimator. This yields the existing links and we would expect that A would be a somewhat sparsely populated matrix. One can see from our previous analysis how this may be the case.

2. Then we calculate estimates for the zeroth state and we enter an estimate for the zeroth concentration matrix.

3. Then we make a measurement and this measurement is used to adjust the initial guess, as well as subsequent guesses.

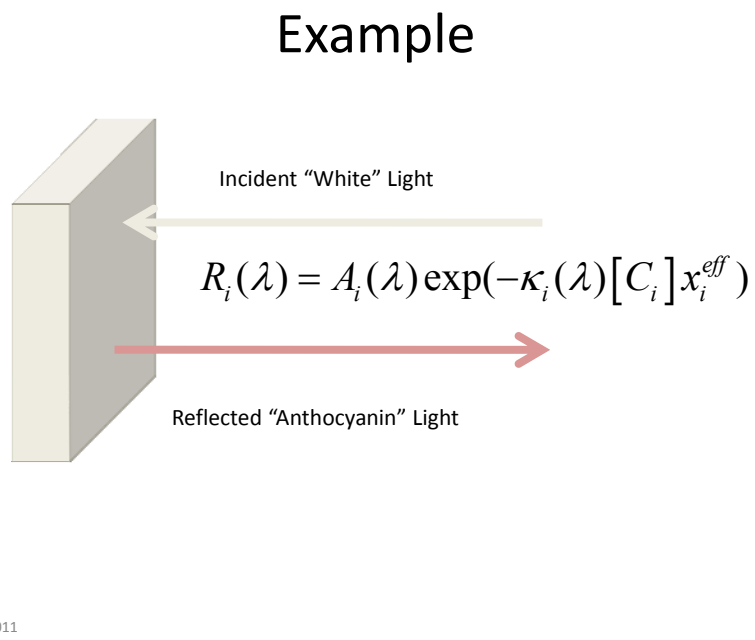
4. Then we change the guesses accordingly as we see whether it is greater or less than the previous guess. If we can drive the answer to zero, namely the difference of the Ax product,

which is the goal of a Newton procedure, then we can achieve convergence. The constant is chosen heuristically to time the convergence.

9.5.2 Measurements

To understand the use of microarray data we look briefly at the issue of reflectance from the surface and relate it to concentrations. It is essential to understand this process since it becomes an integral part of the overall calibration method.

Let us begin with a simple model of reflectance. We look at the Figure below and see a white light impinging on a cell and the light reflected back is seeing at one specific wavelength, frequency, as an attenuated version of what was transmitted at the wavelength. A is the amplitude of the transmission and the exponentially reduced A value is what is reflected. Thus if absorption is in the red and blue as we saw with chlorophyll then we reflect green and that is what we see. This is an application of Beer's Law[124]. Beer's law is a statistical approach to absorption. It reflects what experimentally is obtained and does not provide a detailed analysis as we had been developing in prior sections.



We define the reflect light at a specific frequency, wavelength, as follows:

$$R_i(\lambda) = A_i(\lambda)\exp(-\kappa_i(\lambda)[C_i]x_i^{eff})$$

where [C] is a concentration and x is the effective thickness of the cell.

[124] See Cantor and Schimmel, pp. 60-68.

Here R is the reflected light we see at the wavelength specified and at the ith protein.
A is the incident light amplitude at the wavelength specified. The exponent is Beer's law where C is the concentration of protein I and x the effective depth of that protein.

Now we can write Beer's law for one or two or even more absorbents. We show the case for one and two absorbents as follows:

$$\frac{dR}{R} = -C_k \kappa_k dx$$

or

$$\frac{dR}{R} = -C_k \kappa_k dx - C_{k+1} \kappa_{k+1} dx$$

Note that the reduction in reflected light or in transmitted light is reduced by a result of the additive reduction of separate collisions with separate molecules.

$$R_{Total}(\lambda) = A(\lambda) \exp(-\sum_{i=1}^{N} \kappa_i(\lambda)[C_i]x_i(\lambda))$$

The log of the ratio of intensities is the sum of the weighted concentrations. We assume we know the κ values for each absorbing element at each wavelength. Then we can use the above to estimate the separate concentrations

$$I(\lambda) = \ln \frac{R_{Total}(\lambda)}{A(\lambda)} = -\sum_{i=1}^{N} \kappa_i(\lambda)[C_i]x_i(\lambda)$$

The problem is simply stated. We measure the intensity at say M values of wavelength and this gives us M samples. We then must find values of the [C] which give the best fit to the measurements obtained using the model assumed. That is for every wavelength, we define an error as the difference between the measurement and what the measurement would have been using the estimates of the [C] values and the best [C] values are those which minimize the sum of the squares of these errors. There are M measurements and N concentrations and M is much larger than N. That is:

Choose $[C_n]$ *such that they minimize*

$$\min(\sum_{m=1}^{M} \left(I(m) - \hat{I}(m) \right)^2$$

where

$I(m)$ *is the mth measurement*

and

$$\hat{I}(m) = \sum_{i=1}^{N} \kappa_i(\lambda)[\hat{C}_i]x_i(\lambda)$$

This is an optimization problem which can be solved in many ways. We address some of them in the next section.

9.5.3 Inversion Approaches

We will now look at several general methods of inversion, mostly applied to dynamic system inversions, where we may have dynamics in space and/or time. In many ways this is an example of the Inverse Problem already solved by McGarty (1971):

1. CIE approach: This assumes that one can unravel the exponents of the x,y,z model. The problem is that we will not have an adequate number of degrees of freedom.

2. Splines: This assumes we can generate curves and then separate them and then focus on their coefficients[125].

3. Steepest Descent: This is the incremental approach of best fit. It assumes we are trying to solve an optimization problem.

4. Least Squares Fit: A statistical best fit method.

5. Kalman Filter: This is the statistical solution using steepest descent but with correlation matrices.

6. Matched Filter: This approach assumes we know the waveforms of each absorption curve for each colorant and that we receive a resulting absorption curve which is the sum of all of them, and that we then try to estimate the "amplitudes" of each curve, in effect the concentrations.

9.5.3.1 CIE Approach

We briefly look at using the color data directly. This we call the CE approach since it employs the CIE color structure. We may define the problem as follows:

Let $R(\lambda)$ *be determinable for a given set of* $[C_i]$ *and let*

$\widetilde{R}(\lambda)$ *be the measured received spectrum power and*

$I(\lambda)$ *be the log of the received to incident power*

at the wavelength

Find the set of $[\widehat{C}_i]$, *i=1...N, such that*

$\left(\widetilde{R}(\lambda) - \widehat{R}(\lambda)\right)^2$ *is minimized where* $\widehat{R}(\lambda)$*is the*

estimated received spectral element

[125] See Hildebrand pp. 478-494. The use of splines is an approach which tries to match coefficients of polynomials.

We may also characterize the variables as follows:

Let

$$x(k) = \begin{bmatrix} C_1 \\ . \\ . \\ C_n \end{bmatrix} = x(k+1)$$

and

$$z(k) = c^T(k)x(k) + n(k)$$

where

$$c(k) = \begin{bmatrix} -\kappa_1(k)x_1 \\ . \\ . \\ -\kappa_n(k)x_n \end{bmatrix}$$

and for this case k and λ are identical increments

We now consider three possible approaches.

9.5.3.2 Newton Steepest Descent

Thus for any of the pathways we have described above we can readily apply the model we have developed. The issue will be one of simplification. In the previous quasi steady state analysis we eliminate one reaction as not being significant to the "scale" of the reaction or reactions of interest. We assume it has reached a steady state by the time we enter the second reaction. There are two other approaches; first neglect scale and just deal with the complex nonlinearities and compute results or at the other extreme linearize the system.

Let us apply linearization to the above:

$$\frac{dS}{dt} = \frac{k_2 E_0 S}{K_m + S}$$

let

$$S = S_0 + s$$

where

$s = small\ variation$

then

$$\frac{ds}{dt} = \frac{k_2 E_0 (S_0 + s)}{K_m + S_0 + s} \approx a + bs$$

where

$$a = \frac{k_2 E_0 S_0}{K_m + S_0}$$

$$b = \frac{k_2 E_0}{K_m + S_0}$$

Thus we can look at small variations in such a linear manner.

$g(a) = 0$

is the desired result. Define:

$$A(a) = -\left[\frac{\partial g(a)}{\partial a}\right]^{-1}$$

where we define:

$$\left[\frac{\partial g(a)}{\partial a}\right] = \begin{bmatrix} \frac{\partial g_1}{\partial a_1} & \cdots & \frac{\partial g_1}{\partial a_n} \\ \frac{\partial g_n}{\partial a_1} & \cdots & \frac{\partial g_n}{\partial a_n} \end{bmatrix}$$

and the estimate at sample k+1 is:

$\hat{a}(k+1) = \hat{a}(k) + A(\hat{a}(k))g(\hat{a}(k))$

Note that we use this iterative scheme as one of several means to achieve the result. For each tuple of data we do the following:

$\hat{a}(0) = a^0$, an n x 1 vector guess. Then we use the first data tuple:

$\hat{a}(1) = \hat{a}(0) + A(\hat{a}(0))g((\hat{a}(0))$

where we use the difference:

$a_{k,measured}(0) - \hat{a}(0)$

as the data entry element for each of the elements of a.

The Newton algorithm is but one of many possible algorithms. We know the conditions for Newton convergence. We can also estimate the accuracy of this algorithm as well. We can continue its analysis as follows. The Newton Steepest descent approach is one where we define an optimization and this optimization results in solving a polynomial equation. We then employ an iterative method to solve that equation. We now seek the following:

Find the a such that:

$$\hat{a} = \begin{bmatrix} \hat{a}_1 \\ .. \\ \hat{a}_n \end{bmatrix} = \begin{bmatrix} [C_1] \\ . \\ . \\ [C_N] \end{bmatrix}$$

such that

$$\min\left[\sum_{i=1}^{M}(I_i - \hat{I}_i)^2\right]$$

Let us recall the simple optimization result:

$$h(a) = \left[\sum_{i=1}^{M}(I_i - \hat{I}_i)^2\right]$$

and

$$\frac{\partial h(a)}{\partial a_n} = g_n(a) = 0$$

is the optimal point, so we seek to solve the vector equation:

g(a)=0

We can now state the general solution in terms of Newton's Method[126]:

[126] See Athans et al, Systems, Networks and Computation, Multivariable Methods, McGraw Hill (New York) 1974, pp-115-122.

$g(a) = 0$

is the desired result. Define:

$$A(a) = -\left[\frac{\partial g(a)}{\partial a}\right]^{-1}$$

where we define:

$$\left[\frac{\partial g(a)}{\partial a}\right] = \begin{bmatrix} \frac{\partial g_1}{\partial a_1} & \cdots & \frac{\partial g_1}{\partial a_n} \\ \frac{\partial g_n}{\partial a_1} & \cdots & \frac{\partial g_n}{\partial a_n} \end{bmatrix}$$

and the estimate at sample k+1 is:

$\hat{a}(k+1) = \hat{a}(k) + A(\hat{a}(k))g(\hat{a}(k))$

Note that we use this iterative scheme as one of several means to achieve the result. For each tuple of data we do the following:

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where we use the difference:

$a_{k,measured}(0) - \hat{a}(0)$

as the data entry element for each of the elements of a.

The Newton algorithm is but one of many possible algorithms. We know the conditions for Newton convergence. We can also estimate the accuracy of this algorithm as well.

9.5.3.3 Kalman Filter

The method of estimating the structural elements of the gene expression can be structured using a standard set of methodologies. In particular we use the two approaches. The approach was applied to estimating the constituent chemical concentrations of the upper atmosphere, namely the inversion problem, using transmitted light as the probe mechanism. In this case we seek to estimate the gene expression matrix using the concentrations of secondary chemicals as expressed in color concentrations. This is in many ways a similar problem.

Let us consider a six gene model, two color modifying genes and four control genes, two each. The model is as follows. First is a general linear model for the gene production:

$$\frac{dx(t)}{dt} = Ax(t) + u(t) + n(t)$$

Then the entries are as follows:

$$A = \begin{bmatrix} a_{11}..a_{12}..a_{13}..0..0..0 \\ 0...a_{22}..0...0...0...0 \\ 0...0...a_{33}..0...0...0 \\ 0...0...0...a_{44}..a_{45}..a_{46} \\ 0...0...0...0...a_{55}..0 \\ 0...0...0...0...0...a_{66} \end{bmatrix}$$

and

$$u(t) = \begin{bmatrix} u_1 \\ ... \\ u_6 \end{bmatrix}$$

And we assume a system noise which is white with the following characteristic:

$$E\left[n(t)\right] = 0$$

and

$$E\left[n(t)n(s)\right] = N_0 I\delta(t-s)$$

Now we can define:

$$A = \begin{bmatrix} A_1...0 \\ 0...A_2 \end{bmatrix}$$

Where we have partitioned the matrix into four submatrices. This shows that each gene and its controller are separate. Now we can determine the concentrations of each protein in steady state as follows, neglecting the Gaussian noise element for the time being:

$$\begin{bmatrix} x_1 \\ x_2 \\ x_3 \end{bmatrix} = -A_1^{-1} \begin{bmatrix} u_1 \\ u_2 \\ u_3 \end{bmatrix}$$

and

$$\begin{bmatrix} x_4 \\ x_5 \\ x_6 \end{bmatrix} = -A_2^{-1} \begin{bmatrix} u_4 \\ u_5 \\ u_6 \end{bmatrix}$$

We argue that finding either the matrix elements or their inverse relatives is identical. Thus we focus on the inverse elements. Now the concentrations of the proteins are given by the 2 x 2 vector as follows:

$$\begin{bmatrix} z_1 \\ z_2 \end{bmatrix} = \begin{bmatrix} c_{11}...0...0...0...0...0 \\ 0...0...0...c_{24}...0...0 \end{bmatrix} \begin{bmatrix} x_1 \\ x_2 \\ x_3 \\ x_4 \\ x_5 \\ x_6 \end{bmatrix} = Cx$$

The color model remains the same.

The system model is as follows. Let us begin with a model for the vector a that we seek:

$$\frac{da(t)}{dt} = 0 : where$$

$$a(t) = \begin{bmatrix} a_1 \\ ... \\ a_5 \end{bmatrix}$$

In this case we have assumed a is a 5 x 1 vector but it can be any vector. The measurement system equation is given by:

$$z(t) = g(a,t) + w(t)$$

Where z is an m x 1 vector. In this case however we have for the measurement the following:

$$z(t) = \begin{bmatrix} m_1 \\ m_2 \\ m_3 \\ x_1 \\ ... \\ x_6 \end{bmatrix} = g(a,t) + w(t)$$

We now expand in a Taylor series the above g function:

$$g(a,t) = g(a_0,t) + C(a_0,t)\left[a(t) - a_0(t)\right] +$$

$$\frac{1}{2}\sum_{i=1}^{N} \gamma_i \left[a - a_0\right]^T F_i \left[a - a_0\right] + ...$$

Where we have:

$$C = \begin{bmatrix} \frac{\partial g_1}{\partial a_1} & \cdots & \frac{\partial g_1}{\partial a_n} \\ .. & .. & .. \\ \frac{\partial g_m}{\partial a_1} & \cdots & \frac{\partial g_m}{\partial a_n} \end{bmatrix}$$

Thus we have for the measurement:

$$z(t) = C(t)a(t) + \left[g(a_0) - C(a_0)a_0(t) \right]$$

We now use standard Kalman theory to determine the mean square estimate;

$$\frac{d\hat{a}(t)}{dt} = P(t)C^T(t)K^{-1}(z - C(t)\hat{a}(t))$$

where

$$\frac{dP(t)}{dt} = -P(t)C^T(t)K^{-1}C(t)P(t) +$$

$$\sum_{i=1}^{N} PF_i P\gamma_i^T K^{-1}(z - g(a_0)$$

where

$$K\gamma(t-s) = E\left[w(t)w^T(s) \right]$$

In discrete time we have the equation:

$$\hat{a}(k+1) = \hat{a}(k) + PCK^{-1}\left[z(k) - \hat{z}(k) \right]$$

This is identical to the equation we derived from the Newton method.

9.5.3.4 The Matched Filter Approach

This is a different approach and it is an application of signal detection taken from classic communication theory. It assumes we have N signals and each signal shape is known but the amplitude of the individual signals is not known. Then we ask how we can estimate the amplitude of each signal if what we have is a received signal which is the sum of the N plus noise. We begin this approach as follows:

Let us assume there are two waveforms bounded on an interval [0, T]. Let:

$$s_1(t) = s_1^{orthog}(t) + s_1^{remain}(t)$$

$$s_2(t) = s_2^{orthog}(t) + s_2^{remain}(t)$$

such that

$$\int_0^T s_1^{orthog}(t) s_2^{orthog}(t) dt = 0$$

Now there are three questions which we may pose:

1. Does such a decomposition exist, if so under what terms?

2. What is a constructive way to perform the decomposition?

3. Is there an optimum decomposition such that the "distance between the two orthogonal signals is maximized"?

Namely:

$\exists$ *a set* $\{s_1, s_2\}$
such that

$$\int_0^T s^2{}_1(t) dt = E_1$$

$$\int_0^T s^2{}_2(t) dt = E_2$$

and

$\exists \max$

$$\int_0^T s^{2,orth}{}_1(t) dt = \widetilde{E}_1$$

$$\int_0^T s^{2,orth}{}_2(t) dt = \widetilde{E}_2$$

Let us approach the solution using the theory of orthogonal functions[127]. Now we can specifically use a Fourier series approach. We do the following:

[127] See Sansone, Orthogonal Functions.

Let

$$s_1(t) = \sum_{n=1}^{\infty} s_1^n \cos(\frac{2\pi}{T} nt) + r_1(t)$$

where

$$r_1(t) = s_1(t) - FS\cos$$

and

$$FS\cos = \sum_{n=1}^{\infty} s_1^n \cos(\frac{2\pi}{T} nt)$$

Likewise

Let

$$s_2(t) = \sum_{n=1}^{\infty} s_2^n \sin(\frac{2\pi}{T} nt) + r_2(t)$$

where

$$r_2(t) = s_2(t) - FS\sin$$

and

$$FS\sin = \sum_{n=1}^{\infty} s_2^n \sin(\frac{2\pi}{T} nt)$$

Clearly FS_{cos} and FS_{sin} are orthogonal. The residual functions r are the sin and cos elements respectively of the expansions. We could have just as easily transposed the sin and cos allocations between the two s functions. As to answering the third question we are effectively asking if the r residual functions can be minimized. The answer is not with a Fourier Transform. Then the question would be; is there another set of orthogonal functions which would minimize the residuals, namely:

$$\int_0^T r_1^2(t)dt = R_1$$

and

$$\int_0^T r_2^2(t)dt = R_2$$

are to be minimized. For a Fourier Transform as the orthogonal base we are left with residuals, R, at whatever they may be. However using the Fourier Transform approach we can extract the two signals as follows:

$$P(\lambda) = [C_1]\tilde{s}_1(\lambda) + [C_2]\tilde{s}_2(\lambda) + r_{TOT}(\lambda)$$

Note we can interchange t and λ since they represent the same variable. We now have a "signal" with amplitudes to be determined and a bias which is known. Using standard "signal detection theory" we can readily solve this problem as well. This becomes the "matched filter problem"[128].

What we have sought to accomplish in this paper is to describe color and it generation in plants and to present a set of methods and means to determine the constituents which give rise to those colors. In effect we have created a world view of color, apart from the classic colorimetry approach, and used this and the physical measurements related thereto to affect a method and means to determine concentrations of colorants in flowers.

The simple application of Beer's law and the use of the known spectra of proteins and other colorants allow us to use data from FTS to determine the concentrations of each colorant on literally a cell by cell basis. Beer's law is a simplistic but fairly accurate and consistent method. It would be interesting to explore the details of the transmission of light to a deeper level but the complexity of the cell structure prohibits that at this time.

Having a methodology of the type developed herein we can now more readily examine the genetic pathways and expression systems in the genus Hemerocallis. This paper details multiple ways to ascertain concentrations on a cell by cell basis.

[128] See VanTrees, Detection, Estimation and Modulation Theory. He presents details on this solution.

9.6 ENZYMATIC REACTIONS AND PATHWAYS

In the pathways in the cells one faces not just a simple, albeit structurally complex, network of gene products, but a collection of dynamic enzymatic interactions. We first provide an example, then proceed through the panoply of different reactions and then try to apply the theory to a somewhat complex pathway.

Let us begin with a simple example, the PTEN reaction. We will examine several varying descriptions and then focus on a specific model. The issues here will be fundamentally the following:

1. What is the linkage from gene product to gene product?

2. What is the reaction from gene product to gene product? Is it a simple conversion from one to another, or is it an enzymatic process, and if so what type of enzymatic process.

3. If there are enzymatic reactions and if we know or can assume a form of the enzymatic reaction, then how can we measure the reactions in such a manner so as to determine the constants and validate them.

4. If the reaction is temporal, what are the temporal dynamics? Are there cycles.

5. Is the reaction also spatial, namely do the cells communicate between each other in such a manner so as to communicate changes across the cell matrix? We have examined this phenomenon in other cell environments and there has been recent studies of this phenomenon in melanoma cells. The question is do they also function on cancer and if so what are the dynamics?

6. If we can determine the temporal characteristics then can we look for cycles?

7. If we can determine the dynamics and models, what are the remaining causative factors which initiate the process. For example, what causes the loss of PTEN. We can determine what happens when it is lost, but is it a methylation as some have suggested, and if so is it permanent?

First we examine the pathway and its control of mTOR and cell death. Below is the NCI PTEN control pathway with mTOR[129]. A second example is PTEN controlling apoptosis is also available[130]. Both pathways can be examined in the references attached.

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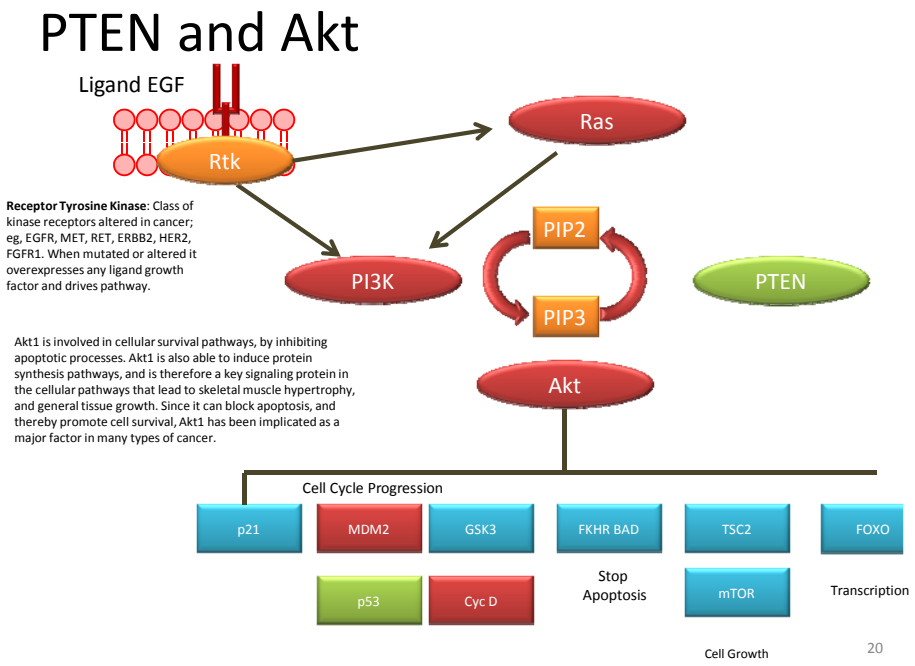
129 http://pid.nci.nih.gov/search/pathway_landing.shtml?what=graphic&jpg=on&pathway_id=100101&source=2&output-format=graphic&ppage=1&genes_a=5728

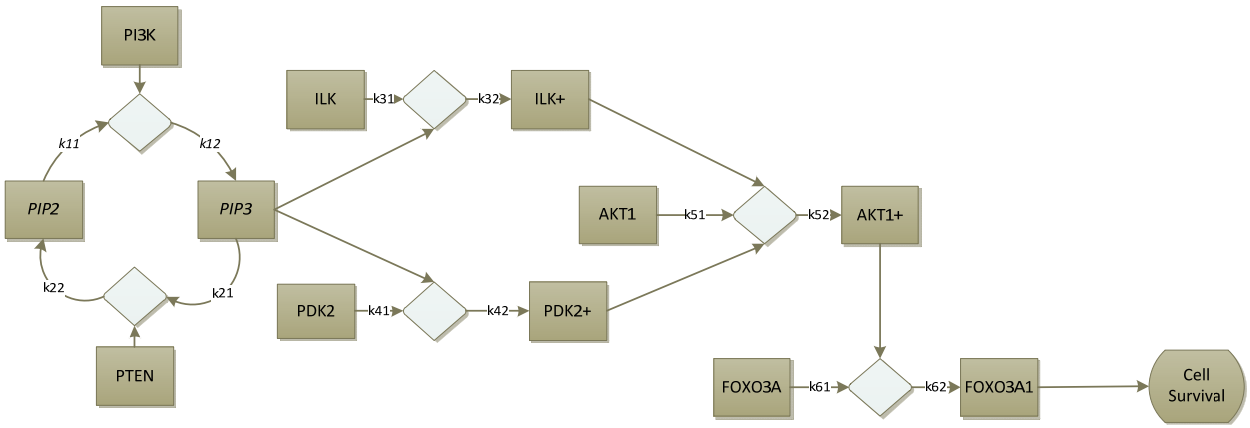
130 http://pid.nci.nih.gov/search/pathway_landing.shtml?pathway_id=100058&source=BioCarta&genes_a=5728&genes_b=&what=graphic&jpg=on&ppage=1

Each step is a reaction and each reaction is facilitated by some enzyme, other protein, which leads to the end result. However since the enzymes are in time varying concentrations and the reactions are temporal and dependent on the specific concentrations, we have a definable but complex dynamic system.

The above reaction can be simplified as follows:



These simplifications are descriptive at best but fail to demonstrate a clear set of reactions, enzymatic, and otherwise. We can show these reactions in the diagram as follows:



These reactions can be described by dynamic reaction models using standard forms. We rely upon Klipp et al for this formalism. We use [X] as the concentration of a product X and we assume that v is some well determined gross reaction rate which may be dependent upon many factors. For simplicity we defer detailed descriptions of the v until necessary. Now we can write a set of reactions using the approach detailed in Klipp et al to show:

$$\frac{d[PIP2]}{dt} = v_{12} - v_{21}$$
$$\frac{d[ILK]}{dt} = v_{31}$$
$$\frac{d[ILK+]}{dt} = v_{13}$$
$$\frac{d[PDK2]}{dt} = v_{41}$$
$$\frac{d[PDK2+]}{dt} = v_{14}$$
$$\frac{d[AKT1]}{dt} = v_{51}$$
$$\frac{d[AKT+]}{dt} = v_{15}$$
$$\frac{d[FOXO3A]}{dt} = v_{61}$$
$$\frac{d[FOXO3A1]}{dt} = v_{16}$$
$$\frac{d[Cell\ Survival]}{dt} = v_{7}$$

The reaction rates, v, are in several cases determined by the enzymatic concentrations of the prior reactions such as:

$$v_{21} = k_{21}[PTEN]$$
$$v_{12} = k_{12}[PI3K]$$
$$v_{42} = k_{42}[PIP3]$$
$$v_{51} = k_{51}[ILK+] + k_{52}[PDK2+]$$
$$v_{61} = k_{61}[AKT1+]$$

where we would have to further analyze the k factor dependence on the constituents of the reaction. The resulting dynamics are quite complex but analyzable. It is immediately evident that oscillations are possible as well as possible instabilities.

The question then is, what are the dynamics of this reaction and given the dynamics of the reaction what are the stabilities or instabilities.

One can assume that if the cell is in some form of homeostatic equilibrium, that perhaps the concentrations of the enzymes, protein, are fluctuating back and forth from some stable set of values. Then, if a perturbation occurs, such as a methylation of PTEN, we seek to determine the new stable points if any exist and the time characteristics of the shifts in those points. An alternative question is how does one measure the time constants and furthermore how does one validate the model employed.

9.6.1 Reaction Models: A Subset

There are many pathways associated with a specific cancer. We discuss a few here in order to develop a few models and structures. The pathway data is from the MSKCC web site which details most of the current gene details as we use the reference of Pestell and Nevalainen also which includes details on most of the specific parts of the pathway.

9.6.2 Reaction Kinetics

Reaction kinetics is a powerful set of chemical dynamics that make the cell function. In this section we review several of the models for reaction kinetics including the Michaelis-Menten model[131]. To start we know that there is a change in a concentration C of some substance and that the concentration may be resulting from one or several competing processes. The following is a general statement of that result;

$$\frac{dC}{dt} = [Synthesis] - [Degradation] - [Phosphorylation]$$
$$+[Dephos] - [Binding] + [Release] + etc$$

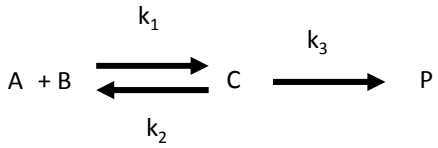
where the brackets indicate concentrations. Much of the current work on modeling pathways is done using the concentration mode. As we have stated earlier, this may or may not be appropriate. Concentration makes sense when we have a large volume with a large number of molecules. Thus the concentration is the average and the standard deviation is small. In a cell, however, we have just a few molecules of any protein, and thus concentration frankly makes little sense. However the concepts and understanding derived from examining this approach does have merit. It provides a base for understanding the differing types and ways in which molecules react.

9.6.2.1 Enzyme Reactions

Now we must provide some specifics to this model to determine its form in detail. The Figure below depicts a typical reaction. This shows how the rate of reaction occurs.

[131] See p. 111 Murray.

Basic Reaction I



C is the intermediate. Assume that the first reaction is in equilibrium. Assume first reaction is so fast and the second is so slow that first is in equilibrium. Then:

$$K = \frac{[C]}{[A][B]}; \quad and \quad K = \frac{k_1}{k_2}$$

$$\frac{d[P]}{dt} = k_3[C] = k_3 K[A][B] = k[A][B] \quad where \quad k = \frac{k_1 k_3}{k_2}$$

Now if we assume that we have the same reaction but there exists a constraint on production, the change in the reaction dynamics is as shown below.

Basic Reaction II

Now consider same reaction, but do NOT assume that the first reaction provides an unlimited amount and assume that there is some form of competition. Then we have:

$$\frac{d[C]}{dt} = k_1[A][B] - k_2[C] - k_3[C]$$

assume that we look at the steady state, derivative equals zero;

$$[C] = \frac{k_1[A][B]}{k_2 + k_3}$$

We will use these basic concepts in the following. We begin with the Michaelis-Menten model and its reactions...

9.6.2.2 Michaelis- Menten Model

Let us begin with a simple enzyme reaction. We start with a source S and a product P with an enzyme E. The reaction is as fo**l**lows:

We first note that this reaction is what controls the production of a protein in the pathway. Namely E is the enzyme and P the resulting product. However, one can also state that the same reaction would occur when the activator and repressor genes produce their proteins and they then modulate the up or down production of the target gene, the one producing E. The intertwining of all of these control and modulation processes is essential if we are to understand the coloring of the flower.

Note that in an enzyme reaction the enzyme E facilitates the reaction and does not end up in any way being part of the product. In fact the enzyme has remained intact at the end of the reaction. Thus the enzyme concentration between the free enzyme and bound portion remains constant. That will be a critical fact in modeling this reaction.

We denote the following as measure of concentrations for this reaction:

s=[S], e=[E], p=[P], c=[SE]

for the respective concentrations. We can now, from the law of mass action, write four equations for the four concentrations. They are as follows[132]:

$$\frac{ds}{dt} = -k_1 es + k_{-1} c$$
$$\frac{de}{dt} = -k_1 es + (k_{-1} + k_2) c$$
$$\frac{dc}{dt} = k_1 es - (k_{-1} + k_2) c$$
$$\frac{dp}{dt} = k_2 c$$

[132] See Murray p. 310.

we assume that the initial conditions are as follows:

$$s(0)=s_0, e(0)=e_0, c(0)=0, p(0)=0$$

From the above differential equations we note the following:

1. p(t) can be calcula6ted if c(t) has been calculated

2. If we add the equations for e and c we find that:

$$\frac{de(t)}{dt}+\frac{dc(t)}{dt}=0$$

Thus we are left with two differential equations:

$$\frac{ds}{dt}=-k_1 s+(k_1+k_{-1})c$$
$$\frac{dc}{dt}=k_1 e_0 s-(k_1+k_{-1}+k_2)c$$

Remember in this notation c is the bound enzyme and source combination, namely SE in the center of the total reaction. Now we want to solve these two equations for s and c. This will yields the results also for e and p since they are as defined as above. We follow Murray by now normalizing the equations. We also note that Murray calculates the change in S not the change in E. Our focus is the change in E over time and space, from that we can obtain the change in S as well. We note from above that we could just as well use the two equations:

$$\frac{ds}{dt}=-k_1 s+(k_1+k_{-1})c$$
$$\frac{de}{dt}=-k_1 e_0 s+(k_1+k_{-1}+k_2)c$$

and then solve for s and e. Now for the normalizations we define:

$$\tau=k_1 e_0 t, u(\tau)=\frac{s(t)}{s_0}, v(\tau)=\frac{c(t)}{c_0}$$
$$\lambda=\frac{k_2}{k_1 s_0}, K=\frac{k_{-1}+k_2}{k_1 s_0}, \varepsilon=\frac{e_0}{s_0}$$

Note that we have use ε as the ratio of initial enzyme to initial source. The use of this will become clear since we are assuming that this is small number since the enzymes concentration is small as compared to the source. This assumption must be looked at closely for this reaction. Then we obtain the following set of equations:

$$\frac{du}{d\tau} = -u + (u + K - \lambda)v$$
$$\varepsilon \frac{dv}{dt} = u - (u + K)v$$
$$u(0) = 1$$
$$v(0) = 0$$

These are nonlinear differential equations which we must solve. However the equations have a singularity which must be dealt with before proceeding. If we want to make the ratio ε small then we need to redefine certain factors. We do this as follows:

$$\sigma = \frac{\tau}{\varepsilon}$$
$$u(\tau, \varepsilon) = U(\sigma, \varepsilon)$$
$$v(\tau, \varepsilon) = V(\sigma, \varepsilon)$$

which yields:

$$\frac{dU}{d\sigma} = -\varepsilon U + \varepsilon(U + K - \lambda)V$$
$$\frac{dV}{d\sigma} = U - (U + K)V$$
$$U(0) = 1$$
$$V(0) = 0$$

This transformation eliminates the singularity about 0. The steady state can be shown as follows:

$$[ES] = \frac{k_1}{k_2 + k_3}[E][S]$$

now let the total enzyme be as follows:

$$[E] + [ES] = [E]_0$$

since only small amount enzyme added, the free free substrate

is almost the total substrate, eg S, then;

$$[ES] = \frac{k_1[E]_0[S]}{k_2 + k_3 + k_1[S]}$$

and we can show that the steady state implies:

$$\frac{d[P]}{dt} = k[E]_0 \text{ where we have } k = \frac{k_3[S]}{K_M + [S]}$$

and we define:

$$K_M \frac{k_2 + k_3}{k_1} \text{ as Michaelis-Menten constant}$$

The above defines the Michaelis-Menten uptake formula. Note the inclusion of the k term which is the rate limiting factor we will see again and again.

There are many other types of reactions and we have discussed them elsewhere. The issue we want to focus on here is that the enzyme is in a reaction of the form where we have a definable time change of enzyme based upon a definable model. That is if [C] is the concentration of an enzyme involved in an enzymatic model we will have a reaction of the type:

$$\frac{\partial[C]}{\partial t} = F([C], t)$$

where F is definable by the reaction. The function F may also consider concentrations of the reactant source and product materials as well. We will use this model in the next section.

$$K = \frac{[C]}{[A][B]};$$

$$\text{and} \quad K = \frac{k_1}{k_2}$$

$$\frac{d[P]}{dt} = k_3[C] = k_3 K[A][B] = k[A][B] \quad \text{where} \quad k = \frac{k_1 k_3}{k_2}$$

A model for the enzyme reactions in a competitive environment has been employed. A method to solve for the Turing space the diffusion model A model to apply the results to a single protein The ability to apply to multiple protein The ability to determine the analysis and the synthesis problem

The Turing model has been discussed earlier. What Turing proposed was that there was some chemical whose concentration made something one way or another. That this something then diffused throughout the organism in some manner and if it was greater in one part than a threshold the morphology was one way and if less the morphology was another. He had no

underlying basis in the current understanding of genetics to put details to his models. We now have that detail.

We know that if we have an activator protein on a secondary pathway then that protein will cause the pathway to become active and create the secondary product, a protein. The more of that protein we have, the greater it concentration, the more secondary product we can get. This is P is the controlling protein concentration, we have:

$$\frac{\partial P_n(x,t)}{\partial t} = H(P_n, A_n, R_n, x, t) + D_P \nabla^2 P_n(x,t)$$

When there are multiple A-R interactions then they add and the net result is an overlapping of the pathway products. The overlays can be shown to create the typical patters in the Petit list. The model allows for an analysis of any tessellated product and also provides a basis for determining what products are achievable as well as how to achieve them, at least at the genetic level. Now we want to build on this model. First we must look at the dynamics of the activator and repressor genes and then we look at the dynamics of the controlling enzyme. Remember that the activator suppressor genes produce products which control the colorant gene. Let us now look at a single cell and look at the tempero-spatial dynamics of the concentrations of the products of the activator and repressor genes, A and S respectively.

Where in this model we have sets of genes and each has activators and repressors. Each gene may activate a separate pathway as we have shown. First we write the model for the controlling enzyme:

$$\frac{\partial P_n(x,t)}{\partial t} = H(P_n, A_n, R_n, x, t) + D_P \nabla^2 P_n(x,t)$$

In the above we show the concentration for the controlling enzyme in a cell for path n. It has a function H which results from a Michaelis-Menten pathway mechanism which we described earlier. From the Michaelis-Menten analysis before we have, if we assume some separate A, R process:

$$H(P,S,SP) = [+k_1 PS - (k_{-1} + k_2)C]Q(A,R)$$

where we had defined PS and C as before and where Q is a function of A and R which either turns on or off the process creating the P reactant. That is if A>R we have a reaction and otherwise we do not.

Thus P is also affected by concentrations of activator and repressor genes, A and R respectively, but in a binary manner. Second, now we write the general model for the activator and repressor product concentrations. As we have just discussed, the pathway activating protein is either on or off. If on we can then calculate its intensity and if off it is irrelevant. For the activator we have:

$$\frac{\partial A_n(x,t)}{\partial t} = F(A_n, R_n, x, t) + D_A \nabla^2 A_n(x,t)$$

and for the suppressor we have:

$$\frac{\partial R_n(x,t)}{\partial t} = G(A_n, R_n, x, t) + D_R \nabla^2 R_n(x,t)$$

Here we have A and R as the relative concentrations of the products of the Activator and Repressor genes. The F and G functions are the mass balance functions for this mix and the additional loss or gain come from the diffusion term. Here we assume that A and R may diffuse at different rates and this fact is key to the oscillations in space and in turn to the tessellation.

9.6.2.3 Basic Kinetics and Reactions

We can now look at a general set of reactions. We assume some reactant X with a concentration [X] and they concentration may increase by means of several different processes. We provide a generic example which is hardly inclusive:

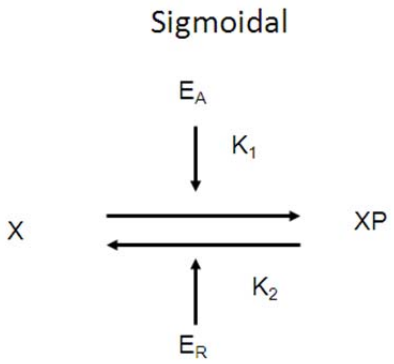
$$\frac{d[X]}{dt} = [Synthesis] - [Degradation] - [Phosphorylation]$$
$$+[Dephosphorylation] - [Binding] + [Release] + etc$$

Now we want to describe a set of such models for the subsequent analysis. Understanding each provides a basis for understanding an integral whole.

9.6.2.4 Sigmoidal Reaction

We now need a model for the interaction functions. We choose the model provided by Conrad and Tyson in Szallasi et al which is termed the phosphorlation-dephosphorlation model or the sigmoidal model[133]. We show its network below. Here we use the enzyme approach with one enzyme, the activator moving the production of the product enzyme and another the repressor enzyme driving the process backward. As we have done with the enzyme case we assume limited amounts and thus we have the
denominators in the equation.

[133] There are a multitude of models here.



In the above reaction we look at the concentration of X. Now X increases via k2 and decreases via k1. However both ER and EA enhance those reactions.

This is the mathematical model we have deployed again using the same reference. This as we have said assumes that we have some form of enzymatic limiting reaction.

$$\frac{d[X]}{dt} = -E_A g([X]) + E_S h([X])$$

and

$$g([X]) = \frac{k_1[X]}{K_{m1} + [X]}$$

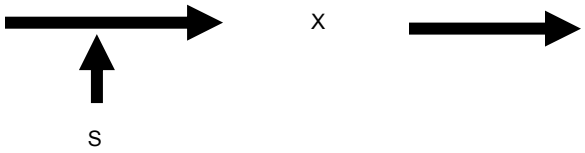
and

$$h([X]) = \frac{k_2\left(x_T - [X]\right)}{K_{m2} + x_T - [X]}$$

The above are also normalized concentrations. We rely upon the recent summary by Baici and the work of McMurray, Schnell as well as Szallasi and his co-authors. This yields a solution for concentrations which is non-linear and which exhibits instabilities and jumps.

9.6.2.5 Synthesis and Degradation

This is an example from Szalassi et al and considers a mRNA, S, which encodes a protein X. We look at the sole reactant and that its reaction is positively driven via S and decays in a manner consistent with the concentration of the reactant X. The reaction is below:



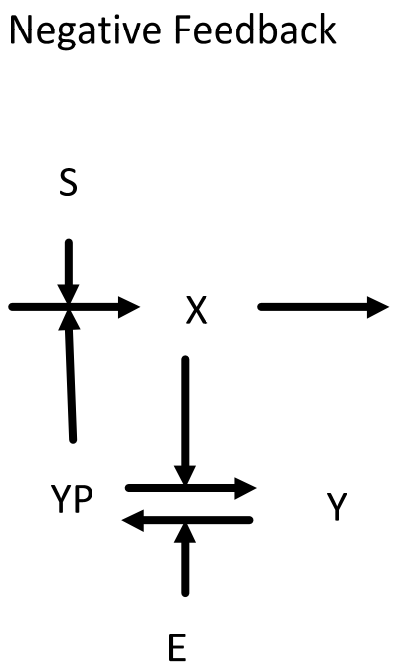
The dynamics of the linearized reaction is also below.

$$\frac{d[X]}{dt} = k_1[S] - k_2[X]$$

Now this reaction is quite simple and it shows exponential growth to a saturation level dictated by S. Here S is the sole outside driver and there is only degradation of [X] due to internal mechanisms.

9.6.2.6 Negative Feedback

The negative feedback model is one of the simpler models as well. We show it below. Here we have two reactions and both affect the other,



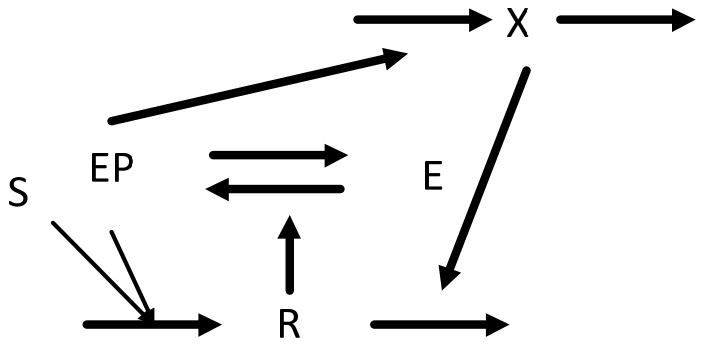
The model then is as follows:

$$\frac{d[X]}{dt} = k_1[S] - k_2[YP][X]$$
$$\frac{d[YP]}{dt} = \frac{k_3[x][[x](y_T - [YP])}{K_{m3} + y_T - [YP]} - \frac{k_4[E][YP]}{K_{m4} + [YP]}$$

9.6.2.7 Activator Inhibitor

This process has an internal process which activates two processes one of which activates the second.

Activator Inhibitor



Here we have a complex process. A substrate S drives the reaction of R as does the enzyme bonded EP and R drives E to EP in a positive loop and EP drives X which in turn drives R to completion.

The dynamics of the process are shown below:

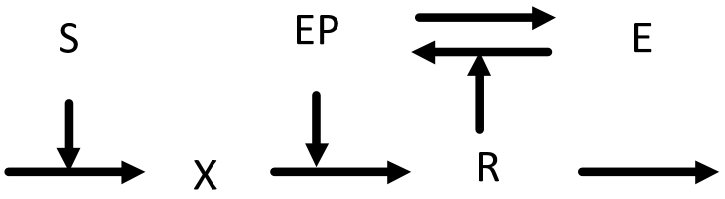
$$\frac{d[R]}{dt} = k_0[EP] + k_1[S] - k_2[X][R]$$
$$\frac{d[X]}{dt} = k_3[EP] - k_4[X]$$

Like the other complex processes this shows cyclic instability.

9.6.2.8 Substrate Depletion

The following shows a process which is initially activated by S then by EP and then the EP is itself activated by the R reactant.

Substrate Depletion



Here we have S producing X and then X produces R which enhances EP which enhances X and the result will be some depletion of S. The dynamics of the process are shown below:

$$\frac{d[X]}{dt} = k_1[S] - \left(k_0^{'} + k_0[EP]\right)[X]$$
$$\frac{d[R]}{dt} = \left(k_0^{'} + k_0[EP]\right)[X] - k_2[R]$$

This system has significant dynamic instabilities. The nature of these instabilities will depend upon the constants and the amount of [S] in the substrate. Note that unlike some earlier models we have limited the dynamics to generally linear in nature, namely first order in each variable.

9.7 CONCLUSION

This chapter presented some basic tools to estimate the reaction coefficients of the reactions we expect in a cell and outside a cell. We focused on three major areas:

1. Cell Dynamics: This was Boolean versus Bayesian, binary versus continuous. The typical pathway model depicts a quasi-binary approach. It shows for example PTEN blocking PI3K type path, a p53 blocking, and so forth. In reality there are many proteins floating around and some block while others may not. On the second hand Bayesian is generally the better approach but the issue is continuous high concentration versus discrete low density. At present there is no know models that the author is aware of which meets all requirements.

2. Identification: This is a critical issue. Here we have the problem of a model, namely a continuous ODE, ordinary differential equation, model with unknown reaction rates. We seek to determine densities, and the resulting cell behavior resulting therefrom. We typically employ a Kalman approach to identification where we can assume with some certainty an underlying ODE system model where we seek only the rate coefficients or constants.

3. Enzymatic Reactions: How the reactions occur is often a major challenge. We generally will linearize everything and work from there. However it is essential to understand the different types of reactions because they result, in the large, in substantial instabilities. Non-linear reactions can and often result in instabilities. Thus we can see unstable results based upon kinetics rather than gene activation or loss of function. We have discussed the issue here but there is at present little actual evidence of this.

9.8 CANCER MODELS FOR UNDERSTANDING, PREDICTION, AND CONTROL

We will now consider what are the essential elements for modeling cancers. The first step is to re-establish the goals of a model and then its structure. Finally we will lead into the interrelationship between a model and the data which is used to justify it.

Many authors have developed models concerning pathways and also cancer. The books by Klipp et al and that of Szlassi et al are excellent overviews of the area with significant detail. The Klipp et al book is a truly superb discussion regarding pathways and modeling alternatives. The books

by Bellomo et al and Wang are directed specifically at cancer modeling but unfortunately they lack adequate pathway dynamics to be of substantial use. Yet they are the only books available within the focused area.

At the core, we want a model which reflects the following qualities:

1. Based Upon Reality: The model must at its core be based upon the known reality. It must conform with what we currently know and understand. Namely it must reflect in its core the elements which we consider critical and the temporal and spatial dynamics of those elements. The model must be based upon a tempero-spatial system of measurable quantities ;linked in some kinetic manner using reasonably well understood processes.

2. Predictability: Any modeling must, if it is to have any credibility, have the ability to predict, to say what will happen, and then to have that prediction validated. Although the ability may be statistical in nature the statistical confidence must be justifiable. We know all too well that many things are correlated, yet not causal, and not predictable.

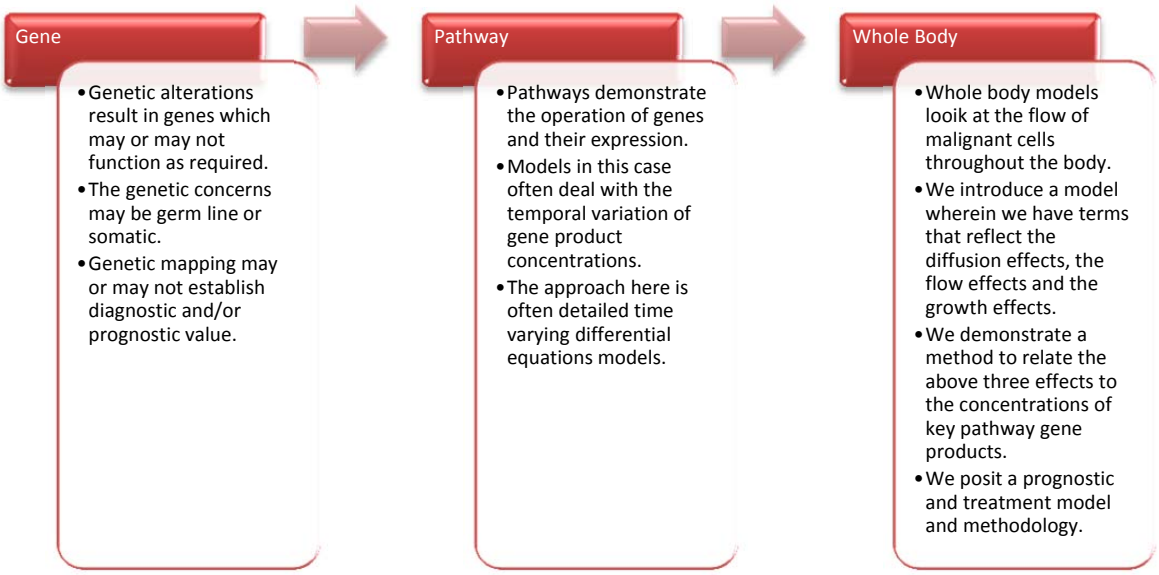
3. Measurable: One must be able to measure and then predict the quantities which make up the model. Many of the modeling systems include proteins but they react in some zero-one format. We know in reality that we have concentrations, or better yet specific numbers of proteins, produced in a cell. Yet we cannot yet measure the number of each of these proteins. We all too often can at best measure their presence or absence. However, is it not the case that it is the excess or the low density of some set of proteins which shift reactions, and that reactions are often concentration dependent.

4. Modellable: We want a system which can be modeled. It must reflect the measurable quantities in space and time and the tempero-spatial dynamics of them, using techniques that we can then use for prediction and validation.

In this paper we examine and analyze several models of cancer. Specifically we look at intracellular, extracellular and full body models. We attempt to establish a linkage between all of them. Many researchers have looked at the gene level, the pathway level and the gross flow of cancer cell level, namely whole body. Connecting them has been complex to say the least.

But herein we look at the pathway level and a whole body level and demonstrate the nexus, physically, and from this we argue that one can construct both prognostic tools as well as methodologies to deal with metastasis.

The following graphic lays out the flow of development and its implications as we detail them herein.


Gene
•Genetic alterations result in genes which may or may not function as required.
•The genetic concerns may be germ line or somatic.
•Genetic mapping may or may not establish diagnostic and/or prognostic value.
Pathway
•Pathways demonstrate the operation of genes and their expression.
•Models in this case often deal with the temporal variation of gene product concentrations.
•The approach here is often detailed time varying differential equations models.
Whole Body
•Whole body models looik at the flow of malignant cells throughout the body.
•We introduce a model wherein we have terms that reflect the diffusion effects, the flow effects and the growth effects.
•We demonstrate a method to relate the above three effects to the concentrations of key pathway gene products.
•We posit a prognostic and treatment model and methodology.

10 CHARACTERISTICS OF CANCER

What are the key characteristics of cancer. As Weinberg notes in the figure below, normal cells have the following functions:

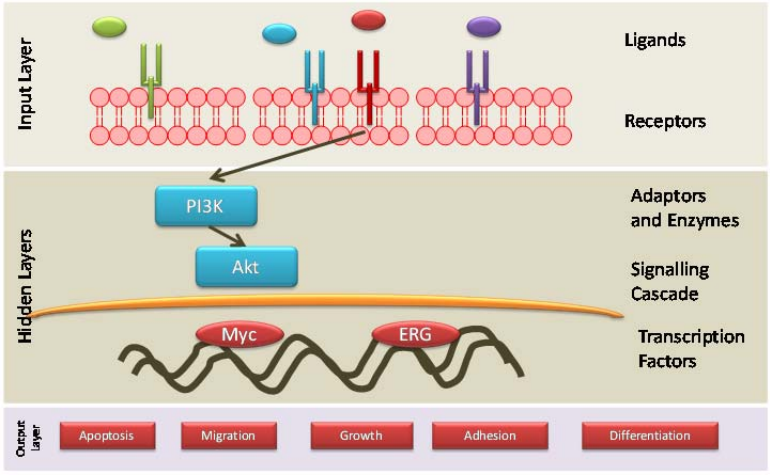
1. Growth: Growth is often distinguished from cell multiplication, or mitosis. Growth is cell expansion, the production of new proteins, the expansion of intra-cellular fluids, and the inclusion of exogenous factors found in normal cell life. In a sense cells consume and cells can get fat! The cells produce and absorb. They consume so they can both grow and duplicate, via simple mitosis.

2. Migration: Cells often have an ability to find their place and stay there. Blood cells flow out into the vascular system, and they tend to stay there until their natural course is complete. Basal cells remain basal as they progress up through the epithelium. Melanocytes remain melanocytes and remain in the basal layers, when benign.

3. Apoptosis: Cells die. They are programmed to do so and they do so in a clean and elegant fashion leaving no mess behind. This is in contrast to cell death with a diseased or damaged cell in which case the immune system must take over and remove the debris.

4. Differentiation: Cells have the ability to differentiate and perform specific functions.

5. Adhesion: Cells stick to adjacent cells, keeping their place in a collection of many other differentiated cells, and performing specific cellular functions.



The following represent several of the characteristics found in cancer cells.

1. Genetic Alterations: The genes in cancer cells are often markedly changed. We have demonstrated that the literature has for melanoma, and for many other cancers, laid out various genetic profiles, some connected with pathway alterations and others just recognized.

2. Cell Cycle Activation: Cells enter mitosis when activated generally y external growth stimuli and in turn activate growth pathways. It is possible that as a result of certain genetic pathway alterations that they may get activated endogenously, rather than exogenously. In either case we have activated the cell cycle and if not controlled the cell will complete mitosis and a doubling will occur. Unlimited cell cycle activation will result in the unlimited growth of cancer cells and the displacement of normal cells and the functions that those cells provide.

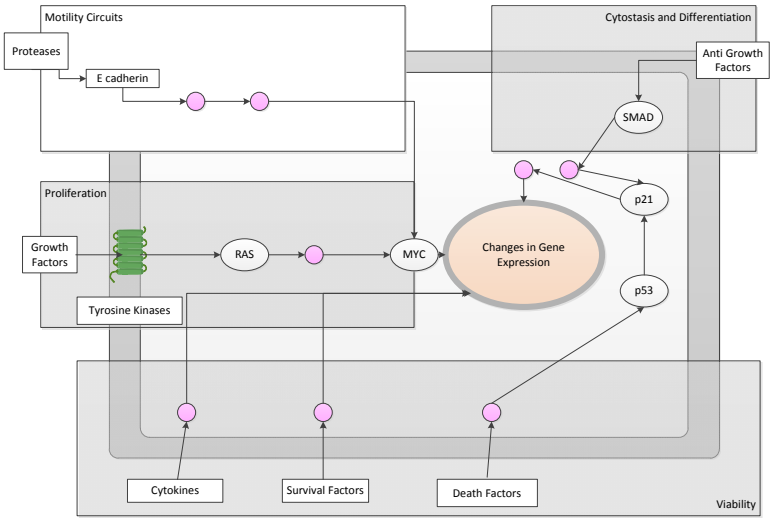
3. Loss of Normal Apoptosis: Loss of normal cell death is a major factor of cancer cells, they just do not die off.

4. Loss of Spatial Stability: The spatial instability is also a known attribute. The melanocyte for example just starts to wander, and then to replicate, and fails to die off through apoptosis.

5. Loss of Specific Functionality: Cancer cells lose all functionality. If a glandular cell, it no longer is. As a malignant melanocyte it no longer produces melanosomes for the skin.

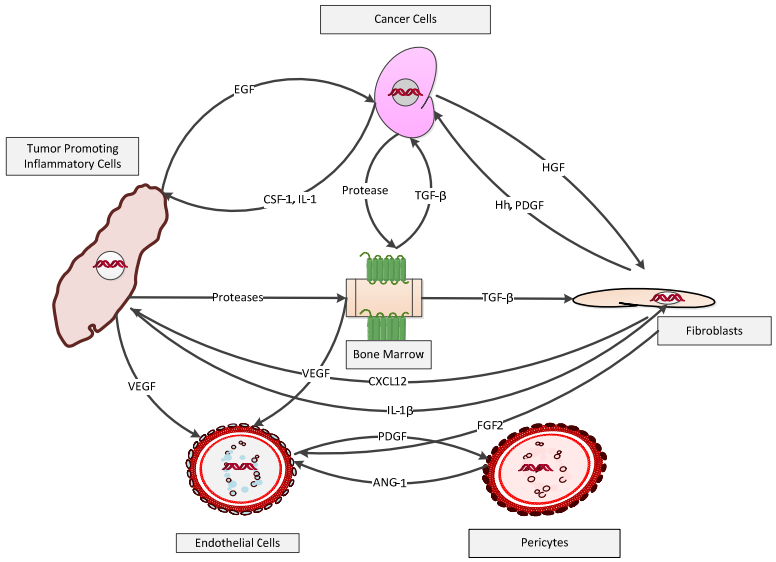
6. Development of Alternative Metabolic Capabilities: This is in effect the Warburg Effect. Namely the metabolism of the cancer cell is anaerobic rather than oxidative.

7. Establishment of a Stem Cell Motif: We have discussed this at length before but it is simply the hypothesis that a small collection of cells control the metastatic process and that this small collection is a clone which may at this point be mutating independently. This implies that the remaining cancer like cells are (i) not capable of separate metastatic, and (ii) if one removes the CSC then one halts the cancer and the remaining cells will go through some form of apoptosis. Also, (iii) the identification of the CSC is separate and apart from all other cells.



10.1 CANCER: AN EVOLVING PUZZLE

Cancer is an evolving puzzle. What we present here is not expected to be definitive. The challenge is that as we learn more about the cell and about cells in environments, it will most likely be reflected in our understanding of cancer cell dynamics. For example, below we have a modification from Weinberg showing the interaction between cancer cells and other cells in the body.



Several recent papers have been published on the details of cancer genetics which make the understanding a continuous process of complexity. Let me first provide a brief précis of how we have progressed to this point:

1. The clone. It has been asserted that almost all cancers begin with a single aberrant cell, the clonal source. From this one cell we have generate everything else. One single cell then replicates in an uncontrolled manner.

2. The Vogelstein Paradigm: The Vogelstein Paradigm (VP) states that the clone is created in some predictable sequence of gene changes and that these changes can be detected and perhaps blocked.

3. The genetic profile: This concept uses the wealth, also excess, of gene mutation data available from microarray analysis to determine "profiles" for various cancers attempting to gain prognostic information as well as "individual" profiling for treatment. In many ways the micro array tool provides "too much data", akin to the comment in Amadeus when the Emperor was asked about Mozart's music, and he remarked "too many notes". Namely the wealth of data is essential but the ability of the human processor is not quite up to it yet.

4. The pathway model: In this case we use pathways as a means to understand what is going wrong in a cell by cell basis. Then we try to block aberrant pathways to have the tumor no longer function as it has to that point. We have argued that this approach has a strong core, namely a model which can be verified and improved, but at the same time it lacks two major factors; (i) is does not deal with intercellular communications well enough, (ii) it does not deal with the issues of what causes the loss of gene activity and homeostasis well enough.

Now there have been several papers in NEJM discussing results on several cancers, kidney and AML, acute myeloid leukemia. Combined they tell and interesting tale. I have already commented on the kidney paper by Gerlinger et al but will add to it in this analysis.

As Gerlinger et al state:

Multiregion genetic analysis of four consecutive tumors provided evidence of intratumor heterogeneity in every tumor, with spatially separated heterogeneous somatic mutations and chromosomal imbalances leading to phenotypic intratumor diversity (activating mutation in MTOR) and uniformity (loss-of-function mutation in SETD2 and PTEN). Of all somatic mutations found on multiregion sequencing, 63 to 69% were heterogeneous and thus not detectable in every sequenced region. Heterogeneous patterns of allelic imbalance were found in all tumors, and ploidy heterogeneity was found in two tumors. Therefore, we found that a single tumor-biopsy specimen reveals a minority of genetic aberrations (including mutations, allelic imbalance, and ploidy) that are present in an entire tumor.

Thus with this study we see significant genetic variability. The sequencing of genetic changes and the expectation of clonal consistency seems to be at variance.

In contrast, to justify the clonal progression, as Walter et al state regarding AML:

A unique aspect of the biology of leukemia is that hematopoietic cells freely mix and recirculate between the peripheral blood and the bone marrow. Clones that persist and grow over time must retain the capacity for self-renewal. Mutations in new clones must confer a growth advantage for them to successfully compete with ancestral clones. The result is that these secondary-AML samples are not monoclonal but are instead a mosaic of several genomes with unique sets of mutations; this mosaic is shaped by the acquisition of serial mutations and clonal diversification. Similarly, recent analysis of de novo AML samples with the use of whole-genome sequencing showed that relapse after chemotherapy is associated with clonal evolution and acquisition of new mutations.

Analysis of individual cancer cells may reveal additional layers of genetic complexity. Recent studies of B-cell acute lymphoblastic leukemia have shown that serial acquisition of cytogenetic abnormalities in that disease most often occurs through a branching hierarchy and only rarely follows a simple linear path…. Our study has several clinical implications.

First, the distinction between the myelodysplastic syndromes and secondary AML currently relies on manual enumeration of bone marrow myeloblasts, a standard that is subject to interobserver bias but nonetheless drives major decisions about treatment for patients with small

differences in myeloblast counts. Ultimately, identifying the patterns of pathogenic mutations and their clonality in bone marrow samples from patients with myelodysplastic syndromes should lead to greater diagnostic certainty and improved prognostic algorithms.

Neither studies presented intracellular pathways models which could be verified as state machines leading to malignant processes nor did they provide any basis for the genetic variations observed. These two factors will be essential in a better understanding of these diseases. However we see strong hematopoietic clonality and non-hematopoietic non-clonality.

The question one may ask is: do the cancer cells as they progress in a metastatic manner do so in a random ever changing manner unconnected from one another or is there some rational underlying physical basis for the changes in a manner in which the cancer has become an alter-organism in the human host? Is cancer a "slime mold" atop the human?

10.2 WHAT DO WE MODEL IN CANCER?

The key question we ask is just what is it we are modeling in cancer cell dynamics. Let us consider some options:

10.2.1 Intracellular Gene Dynamics:

This type of model focuses on the genes, and their behavior. It is basically one where we examine the gene type and its product.

10.2.2 Intracellular Protein Dynamics:

This type of model falls in several subclasses. All begin with protein pathways and the "dynamics" of such pathways. But we have two major subclasses; protein measures and temporal measures. By the former we mean that we can look at the proteins as being on or off, there or not there, or at the other extreme looking at the total number of proteins of a specific type generated and present at a specific time. By the latter, namely the temporal state, we can look at the proteins in some static sense, namely there or not there at some average snapshot instance, or we can look at the details over time, the detailed dynamics. In all cases we look at the intracellular dynamics only.

Let us consider the two approaches.

i. On-Off: In this approach the intracellular relationships are depicted as activators or inhibitors, namely if present they allow or block an element in a pathway. PTEN is a typical example, if present it blocks Akt, if absent it allows Akt to proceed and enter mitosis. p53 is another example for if present we have apoptosis and if absent we fail to have apoptosis. These are simplistic views. This is a highly simplistic view but it does align with the understanding available say with limited microarray techniques. This is an example of the data collection defining what the model is or should be.

ii. Density: This is a more complex model and it does reflect what we would see as reality. The underlying assumptions here are:

a. Genes are continually producing proteins via transcription and translation.

b. Transcription and translation are affected at most by proteins from other genes acting as repressors or activators. There are no other elements affecting the process of transcription and translation. Not that this precludes any miRNA, methylation, or other secondary factors. We shall consider them later. In fact they may often be the controlling factors.

c. The kinetics of protein production can be determined. Namely we know the rate at which transcription and translation occur in a normal cell or even in a variant. That is we know that the production rate of proteins can be given by:

$$\frac{\partial n_j(t)}{\partial t} = \sum_{k=1}^{K} \lambda_k n_k(t)$$

Here we have production rates dependent on the concentration of other proteins. The processes related to consumption are not totally understood (see Martinez-Vincente et al). We understand cell growth, as distinct from mitotic duplication, but the growth of a cell is merely the expansion of what was already in the cell when at the end of its mitotic creation. In contrast, we understand apoptosis, the total destruction of the cell, we also understand that certain proteins flow outside the cell or may be used as cell surface receptors, but the consumption of these is not fully understood. Yet we can postulate:

$$\frac{\partial n_j(t)}{\partial t} = -\sum_{k=1}^{K} \mu_k n_k(t)$$

This is based upon the work of Martinez-Vincente et al which states:

All intracellular proteins undergo continuous synthesis and degradation (Mortimore et al., 1989; Schimke, 1970). This constant protein turnover, among other functions, helps reduce, to a minimum, the time a particular protein is exposed to the hazardous cellular environment, and consequently, the probability of being damaged or altered. At a first sight, this constant renewal of cellular components before they lose functionality may appear a tremendous waste of cellular resources.

However, it is well justified considering the detrimental consequences that the accumulation of damaged intracellular components has on cell function and survival (Goldberg, 2003). Furthermore, protein degradation rather than mere destruction is indeed a recycling process, as the constituent amino acids of the degraded protein are reutilized for the synthesis of new proteins (Mortimore et al., 1989; Schimke, 1970).

The rates at which different proteins are synthesized and degraded inside cells are different and can change in response to different stimuli or under different conditions. This balance between protein synthesis and degradation also allows cells to rapidly modify intracellular levels of

proteins to adapt to changes in the extracellular environment. Proper protein degradation is also essential for cell survival under conditions resulting in extensive cellular damage. In fact, activation of the intracellular proteolytic systems occurs frequently as part of the cellular response to stress (recently reviewed in Cuervo, 2004b; Goldberg, 2003). In this role as 'quality control' systems, the proteolytic systems are assisted by molecular chaperones, which ultimately determine the fate of the damaged/unfolded protein (Fig. 1).

Damaged proteins are first recognized by molecular chaperones, which facilitate protein refolding/repairing. If the damage is too extensive, or under conditions unfavorable for protein repair, damaged proteins are targeted for degradation. Protein degradation is also essential during major cellular remodeling (i.e. embryogenesis, morphogenesis, cell differentiation), and as a defensive mechanism against harmful agents and pathogens (recently reviewed in Cuervo, 2004a; Klionsky, 2005).

We have also discussed this process with regards to the function of ubiquitin, which marks proteins for elimination. As Goldberg states:

Proteins within cells are continually being degraded to amino acids and replaced by newly synthesized proteins. This process is highly selective and precisely regulated[1], and individual proteins are destroyed at widely different rates, with half-lives ranging from several minutes to many days. In eukaryotic cells, most proteins destined for degradation are labelled first by ubiquitin in an energy requiring process and then digested to small peptides by the large proteolytic complex, the 26S proteasome.

Indicative of the complexity and importance of this system is the large number of gene products (perhaps a thousand) that function in the degradation of different proteins in mammalian cells. In the past decade, there has been an explosion of interest in the ubiquitin–proteasome pathway, due largely to the general recognition of its importance in the regulation of cell division, gene expression and other key processes[1]. However, the cell's degradative machinery must have evolved initially to serve a more fundamental homeostatic function — to serve as a quality-control system that rapidly eliminates misfolded or damaged proteins whose accumulation would interfere with normal cell function and viability.

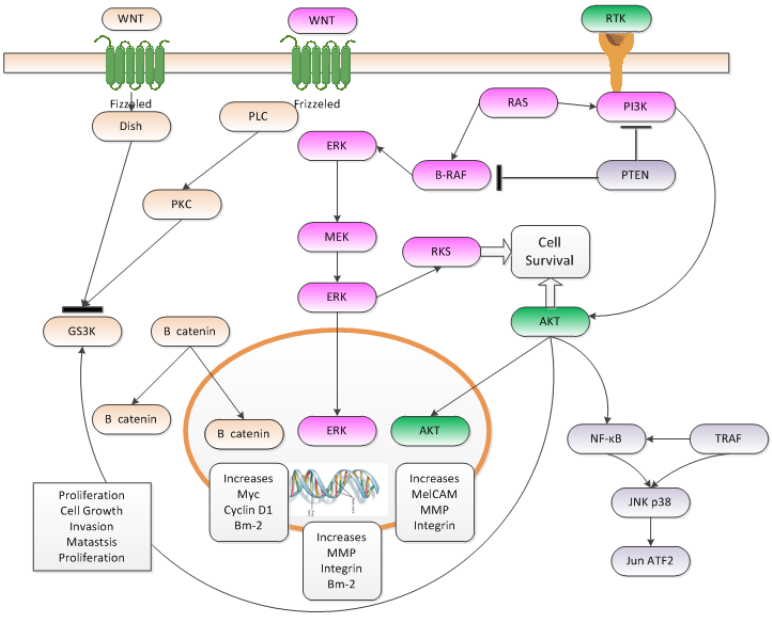
Also we refer to the recent review work of Ciechanover which details the evolution of this understanding.

In contrast the proteins are consumed and thus the negative sign. In toto we have:

$$\frac{\partial n_j(t)}{\partial t} = \sum_{k=1}^{K} (\lambda_k - \mu_k) n_k(t)$$

as a total balance of proteins. This assumes we have a production mechanism for each of the proteins, namely their genes and the activators and repressors as required.

d. Pathway Dynamics must be meaningful. Let us consider the pathway as shown below. This is a typical melanoma pathway we have shown before.



Now let us consider PTEN blocking BRAF and Akt. Now physically it is one molecule of PTEN needed for each molecule of BRAF and PI3K. But what if we have the following:

$$n_{PTEN}(t) < n_{BRAF}(t)$$
$$n_{PTEN}(t) > n_{PI3K}(t)$$

Here we have PTEN blocking some but not all the BRAF and PTEN blocking all the PI3K. At least at time t. Do we have an internal mechanism which then produces even more PTEN? One must see here that we are looking at the actual numbers of PTEN, real numbers reflecting the production and destruction rates. We know for example that if we have a mutated BRAF then no matter how much PTEN we have an unregulated pathway.

Now it is also important to note that this "model" and approach is distinct in ways from classic kinetics, since the classic model assume a large volume and concentrations in determining kinetic reaction rates of catalytic processes. Here we assume a protein binds one on one with another protein to facilitate a pathway.

Thus knowing the dynamics of individual proteins, and knowing the pathways of the proteins, namely the temporary adhesion of a protein, we can determine several factors:

4. The number of free proteins by type
5. The pathways activated or blocked
6. The resultant cellular dynamics based on activated pathways.

It should be noted that we see pathways being turned on and off as we produce and destroy proteins. There is a dynamic process ongoing and it all depends on what would be a stasis level

of proteins by type. The question is; are cells in stasis or are they in a continual mode of regaining a temporary stasis?

This also begs the question, that if as we have argued, that cancer is a loss of stasis due to pathway malfunction, then can this be a process of instability in the course of a normal cell? Namely is there in the dynamics of cell protein counts, unstable oscillator type modes resulting in uncontrolled mitotic behavior. Namely can a cell get locked into an unstable state and start reproducing itself in that state, namely an otherwise normal cell.

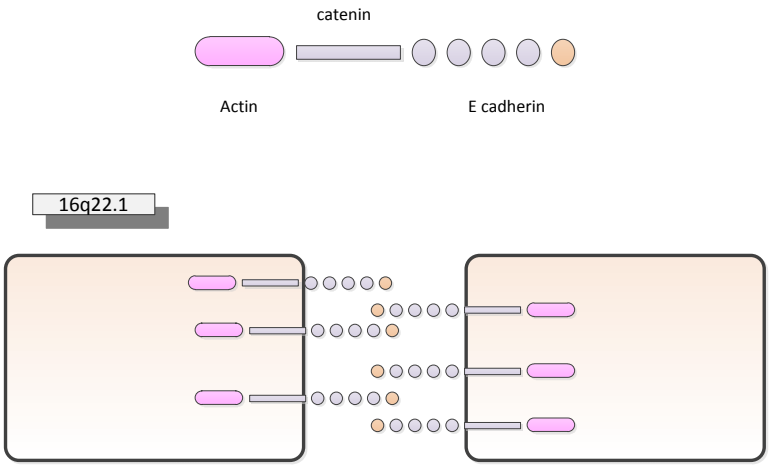
e. Total intracellular dynamics can be modeled yet the underlying processes are still not understood and the required measurements are yet to be determined.

10.2.3 Intercellular Dynamics:

Here we look at the intercellular dynamics as well, not just as a stand-alone model. By this methodology we look at intercellular communications by ligand binding and the resulting activation of the intracellular pathways. We must consider both the intercellular signalling between like cells but also between unlike, such a white cells perhaps as growth factor inhibitors and the like. We also then must consider the spatiodynamics, namely the "movement" of the cells, or in effect the lack of fixedness or specificity of function. This becomes a quite complex problem.

There are two functions we examine here:

a. Intercellular binding or adhesion: E cadherin is one example that we see in melanocytes. Pathway breakdown may result in the malfunctioning of E cadherin.



The above demonstrated E cadherin in melanocyte-keratinocyte localization. The bonds are strong and this stabilizes the melanocyte in the basal layer. If however the E cadherin is compromised then the bond is broken, or materially weakened, and the melanocyte starts to wander. Movement for example above the bottom of the basal layer and upwards is pathognomonic of melanoma in situ. Wandering downward to the dermis becomes a melanoma.

Thus the pathways activating E cadherin production is one pathway essential in the inter-cellular dynamics.

b. Ligand production and receptor production: Here we have cells producing ligands, proteins which venture out of the cell and become signalling elements in the intercellular world. We have the receptor production as well, where we have on the surface of cells, various receptors, also composed of cell generated proteins, which allow for binding sites of the ligands and result in pathway activation of some type. For example various Growth Factors, GF proteins, find their way to receptors, which in turn activate the pathways. Wnt is an example of one of these ligands which we have shown above.

It can also be argued that as ligands are produced and as the "flow" throughout the intercellular matrix, we can obtain effects similar to those in the Turing tessellation models. Namely a single ligand may be present everywhere but density of ligands may vary in a somewhat complex but determinable manner, namely is a wavelike fashion.

Thus, as with intracellular proteins, we have with intercellular ligands the following:

$$q(x,t) = E\left[Number\ of\ ligand\ Proteins; x, t\right]$$

and we would have some dynamic process as:

$$\frac{\partial q(x,t)}{\partial t} = R(x,t)\frac{\partial^2 q(x,t)}{\partial x^2} + \ldots$$

This is akin to the Turing model used in patterning of plants and animals. Namely the concentration of a ligand, and in turn its effect, may be controlled by

10.2.4 Total Cellular Dynamics:

In this case we would want a model which reflects the total body spatiotemporal dynamics This type of models is an ideal which may or may not be achievable. In a simple sense it is akin to diffusion dynamics, viewing the cancer cells as one type of particle and the remaining body cells as another type. The cancer cells have intercellular characteristics specific to cancer and the body cells have functionally specific characteristics. Thus we could ask questions regarding the "diffusion" of cancer cells from a local point to distant points based upon the media in between. The "rate" of such diffusion could be dependent upon the local cells and their ability for example to nourish the cancer cells as well. In this model we could define an average concentration of cancer cells at some position x and time t as:

$$p(x,t) = E\left[Number\ of\ Cancer\ Cells; x, t\right]$$

and we would have some dynamic process as:

$$\frac{\partial p(x,t)}{\partial t} = D(x,t)\frac{\partial^2 p(x,t)}{\partial x^2} + \ldots$$

This is a diffusion like equation and is a whole body equation. Perhaps knowing what the rate of diffusion is on a cell by cell basis may allow one to determine the most likely diffusion path for the malignancy, and in turn direct treatment as well.

This is of course pure speculation since there has been to my knowledge any study in this area. Except one could imagine a system akin to PET scans and the like which would use as input the surface markers from a malignancy and then the body diffusion rates to plot out in space and time the most likely flow of malignant cells and thus plan out treatment strategies. Although this model is speculative we shall return again to it in a final review of such models since it does present a powerful alternative.

This concept of total cellular dynamics is in contradistinction to the intercellular transport. In the total cellular dynamics model we regard the model as one considering the flow of altered cells across an existing body of stable differentiated cells.

We may then ask, what factors drive cancer cells to what locations? One may putatively state that cancer cells will follow the path of least resistance and/or will proceed along "flow lines" consistent with what propagation dynamics they may be influenced by.

10.3 INTRACELLULAR DYNAMICS: AN EXAMPLE

We now consider in more formalistic terms how to develop models. There are three approaches under consideration:

Boolean Networks: The model assumes discrete time on or off nets controlled by the dynamics of the pathway. The Boolean model consists of two elements: first, an assumption that a gene or protein is on or off, second, that the control of that constituent is a Boolean control consisting of AND, OR and NOT Boolean type functions dependent upon precursor constituents. We discuss this in some detail related to PTEN. The Boolean approach is interesting in that it works well at times identifying non-dynamic pathways using massive micro-array data.

Bayesian Networks: The Bayesian model assumes that the value may be analog not binary and that there exists a graph which is what is the desired output. It uses a Bayes model which yields the best fitting graph given data from a microarray and assuming that there are linkages consisting of probabilities in a directed manner. The Bayesian may become time varying as well. As with the Boolean approach it works well with ascertaining linkages and graphs using microarray data and in this case the microarray data may be of an analog nature not just binary.

State Models: These are continuous time and continuous state models of specific pathway concentrations where we employ enzymatic reaction rate models that we have discussed previously. We have used these models in other circumstances and they have proven their worth. Also these models are often more amenable to stochastic effects as well as examination for observability and controllability.

10.3.1 Boolean Networks

The simplest model for pathways is the Boolean model. This has been described in Klipp et al in simple terms. A classification of Boolean Networks has been done in Gershenson. It is based upon three simple assumptions.

First, genes and their products are either on or off, expressed or not expressed. This denies concentration effects.

Thus as an example we may have PTEN and Akt, and they are either 1 or 0. Thus we have the four states, {PTEN, Akt} as 00, 10, 01, 11. Not all may exist, we will show that next.

Second, the effects on a set of gene products on other gene products can be expressed by a Boolean expression.

Thus, for example we have the Binary or Boolean expression:

Akt = NOT PTEN.

Thus if PTEN is 0 then Akt is 1 and likewise if PTEN is 1 then Akt is 0. The states 11 and 00 are not allowed.

Third, this is a discrete time state system where we have the kth instance influencing the k+1 th state.

Thus as above we should state:

Akt(k+1)=NOT PTEN(k)

We may add asynchrony and probabilistic transitions as well to the model.

Now let us formalize the model a bit. We assume that there are N genes or gene products that are of interest. Then the state of the system at any time is:

$$x(k) = \begin{bmatrix} x_1(k) \\ ... \\ x_N(k) \end{bmatrix}$$

where the xs can take on only 0 or 1 in value, on or off.

Now we assume that for each x(k) we have some Boolean equation of the following type:

$$x_j(k+1) = (x_1(k)\, AND\, x_3(k))\, NOT\, x_4(k)\, OR\, x_6(k)$$

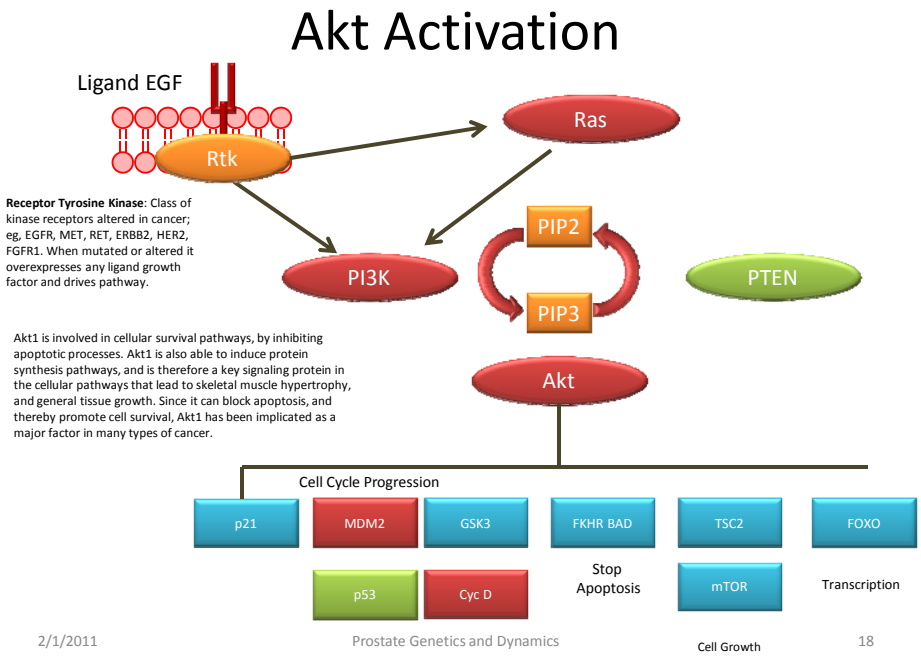
We now ask where did this equation come from. We defer that for the moment. But let us assume that we can find that for every one of the x values. The we have:

$$x(k+1) = F(k+1,k)$$

where

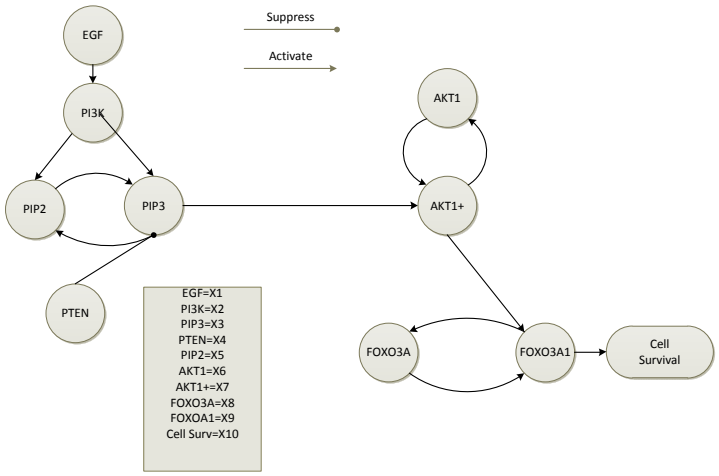
$$F(k+1,k) = \begin{bmatrix} f_1(k+1,k; x_1(k)...x_N(k)) \\ ... \\ f_N(k+1,k; x_1(k)...x_N(k)) \end{bmatrix}$$

Now let us return to our PCa model and look at a simplified set of it as shown below. First, from Lin et al we have for the simple model showing that "*PTEN Decreases AR Protein Levels via Promotion of AR Degradation"* which we show below;



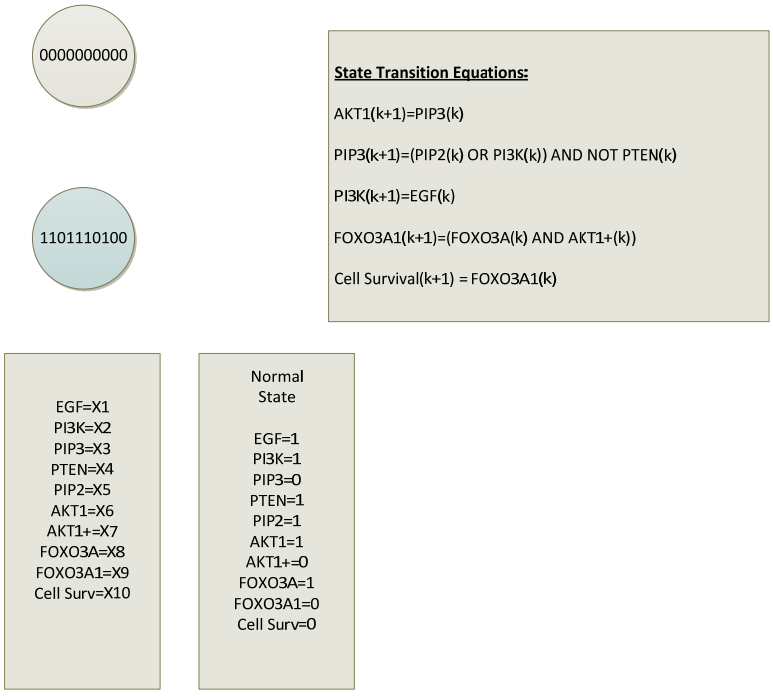
:

Then from the above we can generate a simple model of pathways with some detail as below:



Now in this model we assume that an EGF ligand drives PI3K which moves PIP3 to activate AKT to AKT+1 the activated form which drives FOXO to enhance cell survival. We see 10 states all of which are binary. Ideally we would have 2^{10} possible states, roughly a million. But the state transitions will prohibit this.

The following depict the transition equations, the Boolean f functions from above, as we have somewhat arbitrarily created them, and the stable state.



Note that we can assume that EGF is one and PTEN is 1 and thus PTEN blocks everything. Note also that only 5 state equations are specified. The others are just stable states. We can now take

any states, namely ones where EGF and PTEN go from 0 to 1 or 1 to 0 and see what happens. Note that we have in this model the assumption that PI3K needs EGF. Also if PTEN is on then it suppresses cell growth via FOXO.

One can use microarray technology to validate this model. However it has significant drawbacks.

First it is binary. Real world models are not that way.

Second it is discrete time, again not a reflection of reality.

Third, it assumes binary reactions, and the real reactions are a bit more subtle.

The use of the pathway data is oftentimes effective at the gross level (see Kim et al). Also the ability of correlating pathway models with microarray data is also of significant value. Oftentimes the microarray data itself is binary and thus it maps fairly well onto this model (see Driscoll and Gardner).

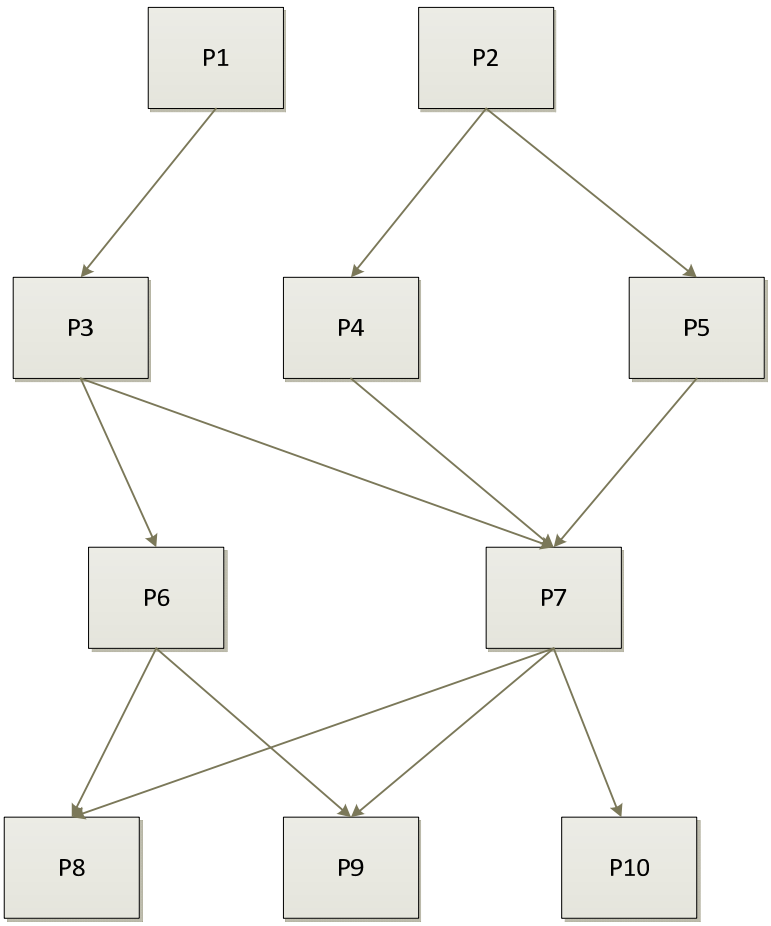
Expansion of this model to non-discrete times and random transitions has been shown in the wok of Shmulevich et al (three papers in 2002, 2002, 2002)

10.3.2 Bayesian Networks

In the development of a Boolean Network we assumed two things; first that the proteins or other relative constituent was either on or off and second that the interaction between constituents was controlled via some Boolean function of AND, OR, NOT type combinations. Now in the Bayesian world we assume Bayes type relationships and then try to infer dependency thereupon.

10.3.2.1 Bayesian Analysis

Let us begin by looking at the following network. We assume that the Ps are influencing the Ps they are connected to in some manner. For simplicity we shall assume they are all positive influences.



Now in a Bayes world we generally have some data and we then try to infer from the data the structure as we see it above.

Let us look at the above flow. Here we have assumed a priori that there are well defined influencing factors. But what if all we had was 10 products and we just thought that they somehow interact. What we want to do is use some logical method to arrive at the chart above. How could we approach this?

Let us assume we have some microarray data. Let us further assume that it is simple microarray data showing genes on or off.

We could write in general the following (see Theodoridis and Koutroumbas, pp 64-68):

$$p(P10, P9, P8, \ldots P1) = p(P10 \mid P9, \ldots P1)\, p(P9 \mid P8, \ldots P1) \ldots p(P2 \mid P1)\, p(P1)$$

Now that does not reflect the graph, G, which we had shown at the top. One may look at the set of all possible graphs, say:

$$\Gamma = \{G_i\}$$

where we have any possible G as a set. We are really interested in the "best" G give say a data set D.

So let us go to the data set, D. What can we use to assist in determining the best possible G. Let us assume we have a microarray. The microarray presents data in one of two forms; active or no effect. Namely the array elements lights up if active and does not light up is not active. Let us assume we have 20 samples and we test for the ten products. We get an array as below. Here red is active.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
P1																				
P2																				
P3																				
P4																				
P5																				
P6																				
P7																				
P8																				
P9																				
P10																				

From this we have a data set given by;

$$d_i = \begin{bmatrix} d_{i,1} \\ \dots \\ d_{i,10} \end{bmatrix}$$

and

$$D = \{d_1, \dots d_N : N = 1, \dots, 20\}$$

We can now look at the problem in a Bayesian sense. Let G be the graph and D the data as we demonstrated above. Now as a Bayes approach we can seek:

$$max\left[log\, P\left[G|D \right] \right] =$$
$$max\left[log\, P\left[D|G \right] + log\, P[G] + c \right]$$

We thus seek to maximize the Bayes score. We seek the G which given the D gives the best result.

Now we can model the system as follows:

$$P_i = \sum_{k=1,\neq i}^{10} a_{i,k} P_k$$

and

$$d_i = b_i P_i$$

The a values are determined by the graph G. We can add noise to the measurement and even to the system. We can then find the set of a that given the D set minimize the measure we stated above. Since we can assume that we have:

$$P_i = \sum_{k=1,\neq i}^{10} a_{i,k} P_k + n_i$$

and

$$d_i = b_i P_i + w_i$$

where they are zero mean Gaussian, then the metric used to attain a minimum on the data set D to determine the graph G is simple, yet a complex calculation.

Namely we have (see Shmulevich and Dougherty):

$$max\left[log\, P\left[G \middle| D \right] \right] =$$

$$max\left[log\, P\left[D \middle| G \right] + log\, P\left[G \right] + c \right]$$

or

$$max\left[-\sum_{k=1}^{10} \sum_{i=1}^{20} \left(d_{i,k} - \hat{d}_{i,k} \right)^2 \right] + log\, P\left[G \right]$$

where we have used the estimated d for the specific graph G being tested. We do this for every possible graph, G. That is a very computationally complex calculation. Indeed it can become non-calculateable.

We demonstrate in the next section that we can measure analog values for the measurements as well as for the desired connections.

10.3.2.2 Microarrays

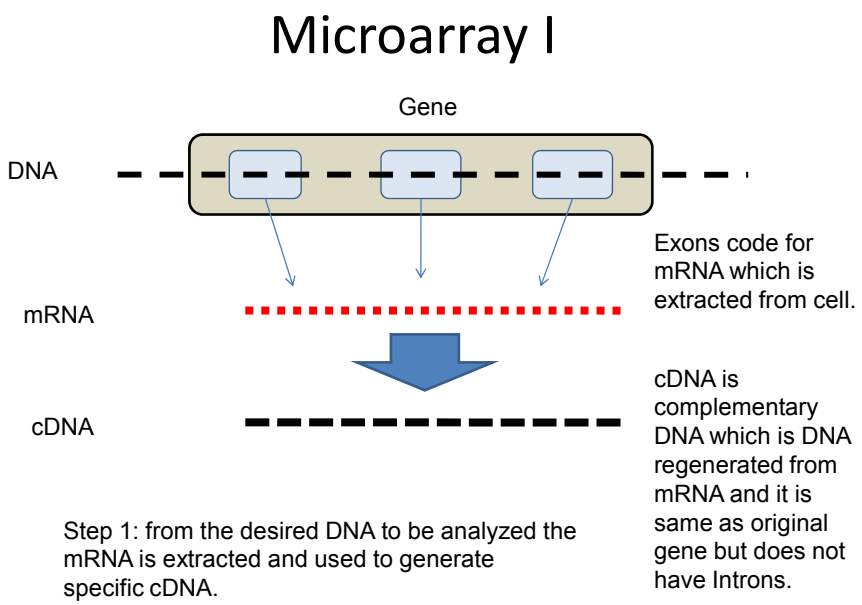
We briefly look at microarrays in some detail since we will use them in the Bayesian case as well in the full system modeling case.

Microarrays are simply a set of matrices where we take samples from many cells and place them in an N by M matrix, say using rows for genes and columns for cell samples. By selecting the rows such that we can place in each row a gene specific binding site, which we can even tag with some visible marker, we can then determine if on a sample by sample basis we have genes being

expressed, namely that they bind at the binding site. It is also possible to attempt to ascertain the concentration of the gene or gene product by having the amount of binding in each sample be reflected by a linear or similar color change. We can say have red for no binding, yellow for some and green for a great deal, or whatever color combination we so desire. Indeed it may then be possible to calibrate for relative concentrations in each cell entry via a colorimetric measurement.

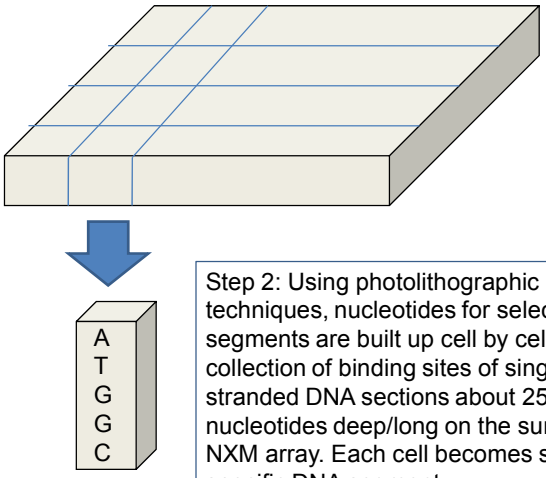
Microarrays is a unique approach which allows for the analysis of millions of samples, it is a marriage of high tech solid state chip technology with DNA bonding. We describe it in the following four steps, each step accompanied by a Figure.

Step 1: The first step in a micro array is the production of cDNA, or complementary DNA. cDNA is that set of nucleotides which account for the encoding of mRNA. It does not include the non-coding regions which are the introns.



Step 2: In a separate environment we make the microcell. This is created in a manner identical to the making of integrated circuits which entails photo-masking techniques. Instead of silicon we used nucleotides. The array has millions of small holes in an array like manner. Each hole we fill with nucleotide, one nucleotide at a time.

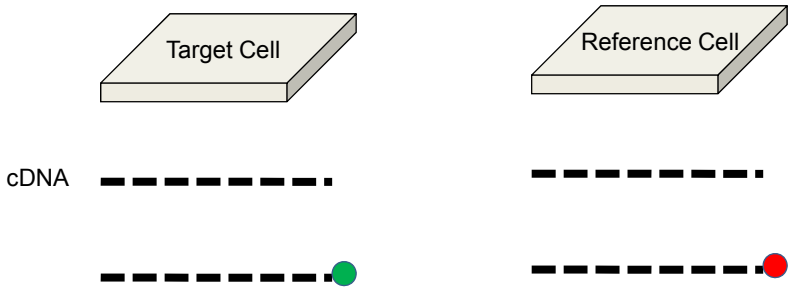
Microarray II



Step 2: Using photolithographic techniques, nucleotides for selected cDNA segments are built up cell by cell creating a collection of binding sites of single stranded DNA sections about 25 nucleotides deep/long on the surface of an NXM array. Each cell becomes sticky for a specific DNA segment.

Step 3: Now we take two DNA samples, one from what we call the Target, the plane we wish to categorize. We then take the segments we collected in step one and tag then with green or red tags, green say for the Target and Red for the Reference.

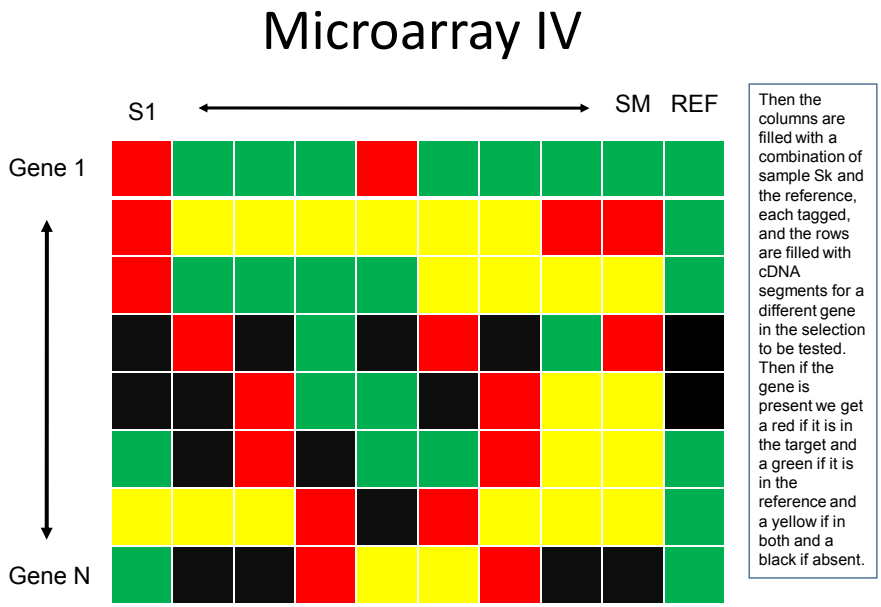
Microarray III



Step 3: For the DNA to be analyzed and a “Reference” target DNA, the mRNA is extracted from each and the cDNA is produced for every gene in the cells to be analyzed, and then it is tagged with a dye which is red for one and green for the other. Typically we tag the target red and Reference green.

Step 4: We then take the samples from the differing plants, one in each column, and look at the array. If the microarray cell has the gene sequence we are seeking to march, and the Target has

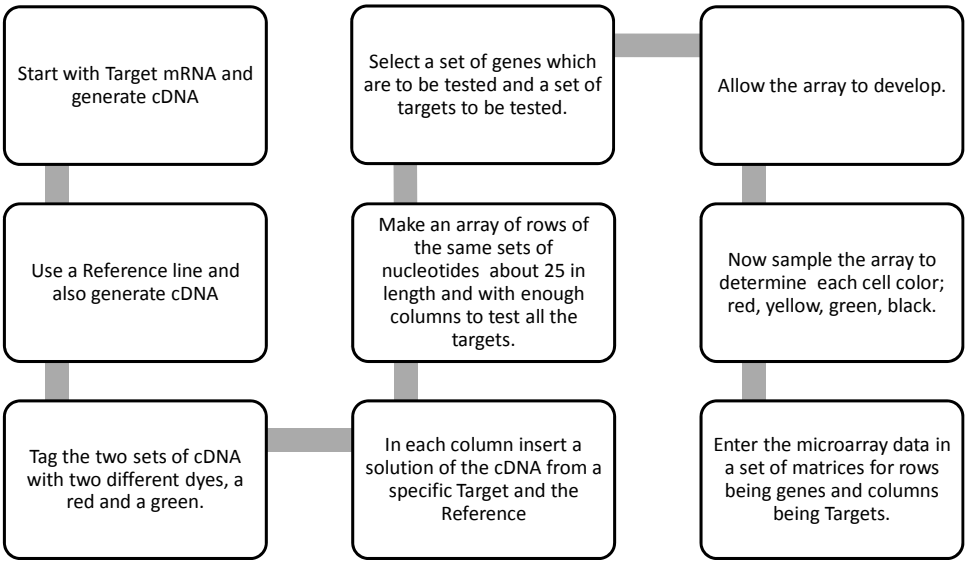
that sequence, it will bond and stick. If the Reference has it, it too will bond. If we just get the Target the cell will be green, if we just get the Reference the cell is red, if we get both the cell turns yellow, and if we have neither the cell is black. The result of a sample scan is shown below.



Now, we even get to try and look at the intensity of the red, green, or yellow. This we can try to see how much is expressed not just whether it is or is not. We will not discuss that here. In the above matrix we can see that many genes are expressed in one or both or none. If we have enough genes than we can argue we have the basis for an exceptionally good means to develop a classification.

In the following Figure we summarize the microarray process.

Microarray Summary



This is a brief summary of microarray technology. We refer the reader to the volume of literature available and referenced to herein.

10.3.2.3 Bayesian Network Summary

The Bayesian network approach can be posed as a general problem. Yet it is often delimited by assuming a priori that certain graphs, G, and no possible, and the minimization or maximization process then is performed over the subset of graphs. There is often still quite a great deal of calculation required.

On the negative side the Bayesian approach as is the case for the Boolean approach does not take into account the underlying chemical dynamics that we have been observing. In addition for most of the pathways we are looking at we already know the key pathway structure and it is the reaction kinetics that we are looking for.

Thus Boolean and Bayesian are useful for identifying pathways whereas the full system model works when the pathway is somewhat well understood and we are looking more for the dynamics and details of the reactions. In a sense they are useful at different stages of the process.

10.3.3 State Dynamic Networks

The state dynamic network model for pathways is the heart of what we have been developing herein. It consists of the following:

1. Intracellular Pathways: The description of pathways within the cell and a description which focuses on concentrations of products and exogenously extracellular products that affect the pathways.

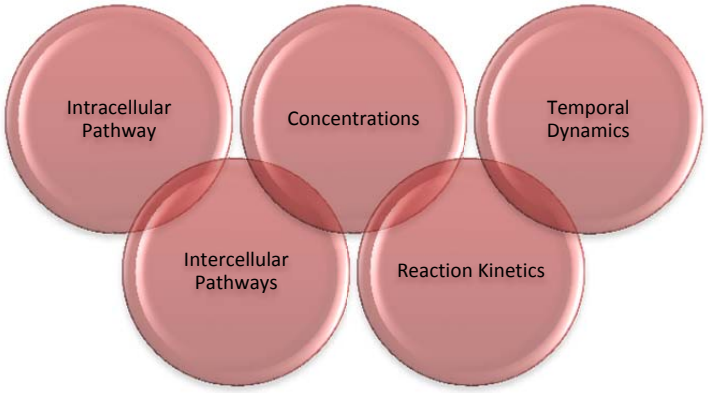
2. Intercellular Pathways: This is the modeling of pathways between and amongst the cells. It is intercellular signalling pathways where receptors and their associated ligands are studied. Again this may generally be focused on concentrations of ligands and similar extracellular and intercellular communicators.

3. Concentrations: The focus is on concentrations of the gene products. The issue here is that it is concentrations which reflect reality and although binding and pathway control may be seen as promoted or inhibited by other proteins, the reality of reaction kinetics is that it is concentrations which are reflective not just single molecule presence or absence. This element is dramatically different from what we see in Boolean or Bayesian models. Here with the modeling with concentrations we are modeling closer to reality.

4. Reactions: Reaction kinetics describes how one substance can control the conversion of another substance into a third. The reaction kinetics are essential to the system dynamics model. The challenge is to know and understand which of the reaction models apply. For example are there rate limiting factors due to concentration limitations of the reaction. However as we have shown before, we are at so low a concentration in a cell that we may very well be dealing with protein to protein bonding, not looking at concentration based reactions. We have not examined this in detail but it will be considered later.

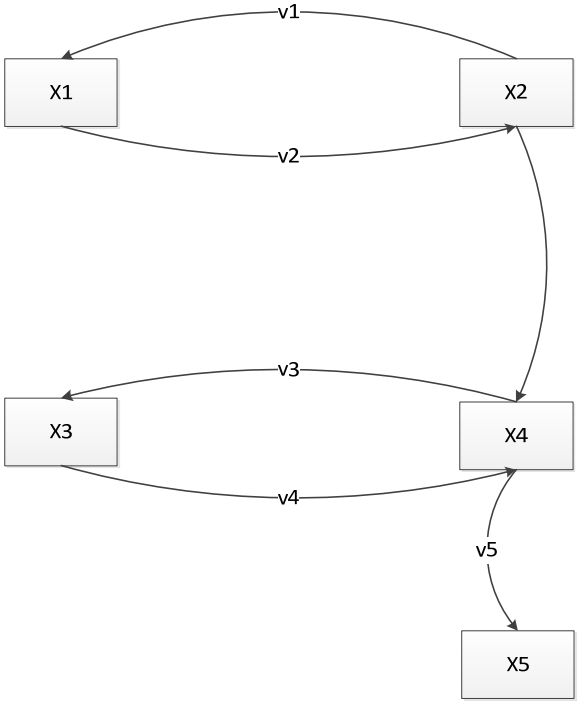
5. Time Variations: The models are all time varying. Thus understanding reaction kinetics from a temporal perspective is essential. This may take our knowledge to the limit and perhaps beyond at the current time.

We graphically demonstrate these factors below:



In order to best understand the system dynamic model we again briefly return to the reaction rate model and its part in defining the reaction rate equations.

Let us consider a simple model as below:



Recall that if we have a pathway with say N=5 constituents then we have the following formula where the rates v are yet to be determined:

$$\frac{d[x_1(t)]}{dt} = v_1 - v_2$$
$$\frac{d[x_2(t)]}{dt} = v_2 - v_1$$
$$\frac{d[x_3(t)]}{dt} = v_4 - v_3$$
$$\frac{d[x_4(t)]}{dt} = v_3 - v_4$$
$$\frac{d[x_5(t)]}{dt} = v_5$$

Now let us return to the rate analysis. We will use the paper by Segel and Slemrod as the basis. This paper presents an excellent analysis of the quasi steady state assumptions for rate and reaction with enzymatic issues as we will encounter.

Assume we have the following reaction:

$$E + S \rightleftarrows C \rightarrow E + P$$

We further assume that the forward rate from E+S to C us k_1 and the reverse is k_{-1} and the forward rate from S to E+P is k_2. Then we have the following:

$$\frac{dE}{dt} = -k_1 ES + k_{-1} C + k_2 C$$
$$\frac{dS}{dt} = -k_1 ES + k_{-1} C$$
$$\frac{dC}{dt} = k_1 ES - k_{-1} C - k_2 C$$
$$\frac{dP}{dt} = k_2 C$$

Note that as we have discussed before the equations are nonlinear due to the product terms. We could linearize them by assuming that we are dealing with small changes or we can use the quasi steady state assumptions which reduces the equations yet keeps the nonlinearity. We shall do the later. Also note the mapping of the above specific reactions to the generalized equations we have used above. Also note that when we have two constituents using an enzyme we see the enzyme as a single combine entity and the initial components as two separate ones. Also we have reaction rates thus dependent to the product of the concentrations on each side of the reaction.

Now let us proceed with the enzymatic reaction. We can restrict the analysis to the following conditions:

$$E(0) = E_0$$
$$S(0) = S_0$$
$$C(0) = 0$$
$$P(0) = 0$$

This is a simple defined initial state when we have just the initial entities which we desire to react. But we also can readily show:

$$E(t) + C(t) = E_0$$

Thus we can simplify the result to what we have below by elimination:

$$\frac{dS}{dt} = -k_1 (E_0 - C0S + k_{-1} C$$
$$\frac{dC}{dt} = k_1 (E_0 - C)S - k_{-1} C$$
$$S(0) = S_0$$
$$C(0) = 0$$

These are still rather complex differential equations and we will now assume that the rates of each are dramatically different. Namely one proceeds quite quickly and the other slowly. That being the case we can thus assume that one is steady state and the other dominates the observable time variation. This is in essence the quasi steady state assumption. This assumption is:

$$\frac{dC}{dt} \approx 0$$

Then we obtain:

$$C = \frac{E_0 S}{K_m + S}$$

where

$$K_m = \frac{k_{-1} + k_2}{k_1}$$

and finally we have the single differential equation for S, which is:

$$\frac{dS}{dt} = \frac{k_2 E_0 S}{K_m + S}$$

This can be applied to the above initial generic form depending upon what the reaction is. Simple reactions do not involve an enzymatic catalyst as we have shown here but in general we will always be dealing with some nonlinearities.

The above deals with four of the five elements we discussed previously: intracellular pathway which was given, rate reactions, concentrations and temporal dynamics.

Thus for any of the pathways we have described above we can readily apply the model we have developed. The issue will be one of simplification. In the previous quasi steady state analysis we eliminate one reaction as not being significant to the "scale" of the reaction or reactions of interest. We assume it has reached a steady state by the time we enter the second reaction. There are two other approaches; first neglect scale and just deal with the complex nonlinearities and compute results or at the other extreme linearize the system.

Let us apply linearization to the above:

$$\frac{dS}{dt} = \frac{k_2 E_0 S}{K_m + S}$$

let

$$S = S_0 + s$$

where

$s = small\ variation$

then

$$\frac{ds}{dt} = \frac{k_2 E_0 (S_0 + s)}{K_m + S_0 + s} \approx a + bs$$

where

$$a = \frac{k_2 E_0 S_0}{K_m + S_0}$$

$$b = \frac{k_2 E_0}{K_m + S_0}$$

Thus we can look at small variations in such a linear manner.

$g(a) = 0$

is the desired result. Define:

$$A(a) = -\left[\frac{\partial g(a)}{\partial a}\right]^{-1}$$

where we define:

$$\left[\frac{\partial g(a)}{\partial a}\right] = \begin{bmatrix} \frac{\partial g_1}{\partial a_1} & \cdots & \frac{\partial g_1}{\partial a_n} \\ \frac{\partial g_n}{\partial a_1} & \cdots & \frac{\partial g_n}{\partial a_n} \end{bmatrix}$$

and the estimate at sample k+1 is:

$\hat{a}(k+1) = \hat{a}(k) + A(\hat{a}(k))g(\hat{a}(k))$

Note that we use this iterative scheme as one of several means to achieve the result. For each tuple of data we do the following:

$\hat{a}(0) = a^0$, an n x 1 vector guess. Then we use the first data tuple:

$\hat{a}(1) = \hat{a}(0) + A(\hat{a}(0))g((\hat{a}(0))$

where we use the difference:

$a_{k,measured}(0) - \hat{a}(0)$

as the data entry element for each of the elements of a.

The Newton algorithm is but one of many possible algorithms. We know the conditions for Newton convergence. We can also estimate the accuracy of this algorithm as well.

10.3.3.1 Kalman Filter

The method of estimating the structural elements of the gene expression can be structured using a standard set of methodologies. In particular we use the two approaches. The approach was applied to estimating the constituent chemical concentrations of the upper atmosphere, namely the inversion problem, using transmitted light as the probe mechanism. In this case we seek to estimate the gene expression matrix using the concentrations of secondary chemicals as expressed in color concentrations. This is in many ways a similar problem.

Let us consider a six gene model, two color modifying genes and four control genes, two each. The model is as follows. First is a general linear model for the gene production:

$$\frac{dx(t)}{dt} = Ax(t) + u(t) + n(t)$$

Then the entries are as follows:

$$A = \begin{bmatrix} a_{11}..a_{12}..a_{13}..0..0..0 \\ 0...a_{22}..0...0...0...0 \\ 0...0...a_{33}..0...0...0 \\ 0...0...0...a_{44}..a_{45}..a_{46} \\ 0...0...0...0...a_{55}..0 \\ 0...0...0...0...0...a_{66} \end{bmatrix}$$

and

$$u(t) = \begin{bmatrix} u_1 \\ ... \\ u_6 \end{bmatrix}$$

And we assume a system noise which is white with the following characteristic:

$$E\left[n(t)\right] = 0$$

and

$$E\left[n(t)n(s)\right] = N_0 I\delta(t-s)$$

Now we can define:

$$A = \begin{bmatrix} A_1...0 \\ 0...A_2 \end{bmatrix}$$

Where we have partitioned the matrix into four submatrices. This shows that each gene and its controller are separate. Now we can determine the concentrations of each protein in steady state as follows, neglecting the Gaussian noise element for the time being:

$$\begin{bmatrix} x_1 \\ x_2 \\ x_3 \end{bmatrix} = -A_1^{-1} \begin{bmatrix} u_1 \\ u_2 \\ u_3 \end{bmatrix}$$

and

$$\begin{bmatrix} x_4 \\ x_5 \\ x_6 \end{bmatrix} = -A_2^{-1} \begin{bmatrix} u_4 \\ u_5 \\ u_6 \end{bmatrix}$$

We argue that finding either the matrix elements or their inverse relatives is identical. Thus we focus on the inverse elements. Now the concentrations of the respective proteins are given by the 2 x 2 vector as follows:

$$\begin{bmatrix} z_1 \\ z_2 \end{bmatrix} = \begin{bmatrix} c_{11}...0...0...0...0...0 \\ 0...0...0...c_{24}...0...0 \end{bmatrix} \begin{bmatrix} x_1 \\ x_2 \\ x_3 \\ x_4 \\ x_5 \\ x_6 \end{bmatrix} = Cx$$

The color model remains the same.

The system model is as follows. Let us begin with a model for the vector a that we seek:

$$\frac{da(t)}{dt} = 0 : where$$

$$a(t) = \begin{bmatrix} a_1 \\ ... \\ a_5 \end{bmatrix}$$

In this case we have assumed a is a 5 x 1 vector but it can be any vector. The measurement system equation is given by:

$$z(t) = g(a,t) + w(t)$$

Where z is an m x 1 vector. In this case however we have for the measurement the following:

$$z(t) = \begin{bmatrix} m_1 \\ m_2 \\ m_3 \\ x_1 \\ \dots \\ x_6 \end{bmatrix} = g(a,t) + w(t)$$

We now expand in a Taylor series the above g function:

$$g(a,t) = g(a_0,t) + C(a_0,t)\left[a(t) - a_0(t)\right] +$$
$$\frac{1}{2}\sum_{i=1}^{N} \gamma_i \left[a - a_0\right]^T F_i \left[a - a_0\right] + \dots$$

Where we have:

$$C = \begin{bmatrix} \frac{\partial g_1}{\partial a_1} & \dots & \frac{\partial g_1}{\partial a_n} \\ .. & .. & .. \\ \frac{\partial g_m}{\partial a_1} & \dots & \frac{\partial g_m}{\partial a_n} \end{bmatrix}$$

Thus we have for the measurement:

$$z(t) = C(t)a(t) + \left[g(a_0) - C(a_0)a_0(t)\right]$$

We now use standard Kalman theory to determine the mean square estimate;

$$\frac{d\hat{a}(t)}{dt} = P(t)C^T(t)K^{-1}(z - C(t)\hat{a}(t))$$
where
$$\frac{dP(t)}{dt} = -P(t)C^T(t)K^{-1}C(t)P(t) +$$
$$\sum_{i=1}^{N} PF_iP\gamma_i{}^T K^{-1}(z - g(a_0)$$
where
$$K\gamma(t-s) = E\left[w(t)w^T(s)\right]$$

In discrete time we have the equation:

$$\hat{a}(k+1) = \hat{a}(k) + PCK^{-1}\left[z(k) - \hat{z}(k)\right]$$

This is identical to the equation we derived from the Newton method.

10.3.3.2 The Matched Filter Approach

This is a different approach and it is an application of signal detection taken from classic communication theory. It assumes we have N signals and each signal shape is known but the amplitude of the individual signals is not known. Then we ask how we can estimate the amplitude of each signal if what we have is a received signal which is the sum of the N plus noise. We begin this approach as follows:

Let us assume there are two waveforms bounded on an interval [0, T]

Let

$$s_1(t) = s_1^{orthog}(t) + s_1^{remain}(t)$$

$$s_2(t) = s_2^{orthog}(t) + s_2^{remain}(t)$$

such that

$$\int_0^T s_1^{orthog}(t) s_2^{orthog}(t) dt = 0$$

Now there are three questions which we may pose:

1. Does such a decomposition exist, if so under what terms?

2. What is a constructive way to perform the decomposition?

3. Is there an optimum decomposition such that the "distance between the two orthogonal signals is maximized"?

Namely:

$\exists$ *a set* $\{s_1, s_2\}$

such that

$$\int_0^T s^2{}_1(t)dt = E_1$$

$$\int_0^T s^2{}_2(t)dt = E_2$$

and

$\exists \max$

$$\int_0^T s^{2,orth}{}_1(t)dt = \widetilde{E}_1$$

$$\int_0^T s^{2,orth}{}_2(t)dt = \widetilde{E}_2$$

Let us approach the solution using the theory of orthogonal functions[134]. Now we can specifically use a Fourier series approach. We do the following:

Let

$$s_1(t) = \sum_{n=1}^{\infty} s_1^n \cos(\frac{2\pi}{T} nt) + r_1(t)$$

where

$$r_1(t) = s_1(t) - FS\cos$$

and

$$FS\cos = \sum_{n=1}^{\infty} s_1^n \cos(\frac{2\pi}{T} nt)$$

Likewise

Let

$$s_2(t) = \sum_{n=1}^{\infty} s_2^n \sin(\frac{2\pi}{T} nt) + r_2(t)$$

where

$$r_2(t) = s_2(t) - FS\sin$$

and

$$FS\sin = \sum_{n=1}^{\infty} s_2^n \sin(\frac{2\pi}{T} nt)$$

[134] See Sansone, Orthogonal Functions.

Clearly FS_{cos} and FS_{sin} are orthogonal. The residual functions r are the sin and cos elements respectively of the expansions. We could have just as easily transposed the sin and cos allocations between the two s functions. As to answering the third question we are effectively asking if the r residual functions can be minimized. The answer is not with a Fourier Transform. Then the question would be; is there another set of orthogonal functions which would minimize the residuals, namely:

$$\int_0^T r_1^2(t)dt = R_1$$

and

$$\int_0^T r_2^2(t)dt = R_2$$

are to be minimized. For a Fourier Transform as the orthogonal base we are left with residuals, R, at whatever they may be. However using the Fourier Transform approach we can extract the two signals as follows:

$$P(\lambda) = [C_1]\tilde{s}_1(\lambda) + [C_2]\tilde{s}_2(\lambda) + r_{TOT}(\lambda)$$

Note we can interchange t and λ since they represent the same variable. We now have a "signal" with amplitudes to be determined and a bias which is known. Using standard "signal detection theory" we can readily solve this problem as well. This becomes the "matched filter problem"[135].

What we have sought to accomplish in this paper is to describe color and it generation in plants and to present a set of methods and means to determine the constituents which give rise to those colors. In effect we have created a world view of color, apart from the classic colorimetry approach, and used this and the physical measurements related thereto to affect a method and means to determine concentrations of colorants in flowers.

The simple application of Beer's law and the use of the known spectra of the proteins allow us to use data from FTS to determine the concentrations of each colorant on literally a cell by cell basis. Beer's law is a simplistic but fairly accurate and consistent method. It would be interesting to explore the details of the transmission of light to a deeper level but the complexity of the cell structure prohibits that at this time.

Having a methodology of the type developed herein we can now more readily examine the genetic pathways and expression. This paper details multiple ways to ascertain concentrations on a cell by cell basis.

[135] See VanTrees, Detection, Estimation and Modulation Theory. He presents details on this solution.

10.4 TOTAL CELLULAR DYNAMICS: AN EXAMPLE

The concept of a model of Total Cellular Dynamics is somewhat innovative. It focuses on the movement of the cancer cells throughout the body. We will consider three possible possibilities:

1. No Stem Cells

2. Stem Cells but Fixed at Initial Location

3. Stem Cells which are mobile.

In Case 1 all malignant cells are clones of each other at least at the start. As the malignant cells continue through mitosis additional mutations are likely so that after a broad set of mitotic divisions we have a somewhat heterogeneous set of malignant cells, some more aggressive than others. As with most such cancer cells they also produce ligand growth factors which stimulate each other and result in the cascade of unlimited growth and duplication.

In Case 2 we assume that there was a single cell which mutated and that this becomes the CSC. The CSC replicates producing one CSC for self-replication and TICs which migrate. We assume that the CSC may from time to time actually double, but not at the mitosis rate of the base. Furthermore we assume the CSC sends out growth factors, GF, to the TICs. The GF flow outward in a wave like manner from the somewhat position stabilized CSCs to the TICs which are mobile and both diffuse and flow throughout the body. The GF must find the TICs which become a distant metastasis.

In Case 3 in contrast to Case 2, we assume mobile CSC and thus the CSCs also flow according to some set of rules.

10.4.1 Total Cellular Dynamics Models

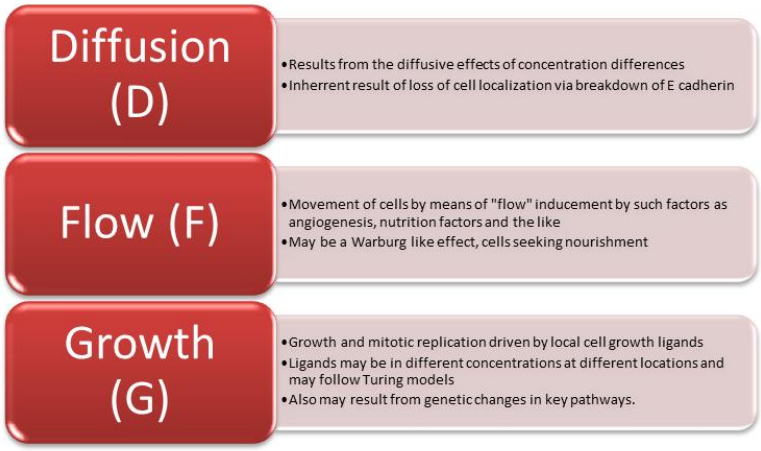
Now depending on the case we assume we can model the flow of cancer cells according to some simple dynamic distributed models[136]. Thus we could have:

$$\frac{\partial p(x,t)}{\partial t} = D(x,t)\frac{\partial^2 p(x,t)}{\partial x^2} + E(x,t)\frac{\partial p(x,t)}{\partial x} + F(x,t)p(x,t)$$

This provides diffusion, flow, and rate elements. The rate term, the F term, is a rate of change in time at a certain location and time specific. It is the duplication rate at that specific location due to the normal mitotic change. The last term may be both pathway and environment driven.

Now this description has certain physical realities.

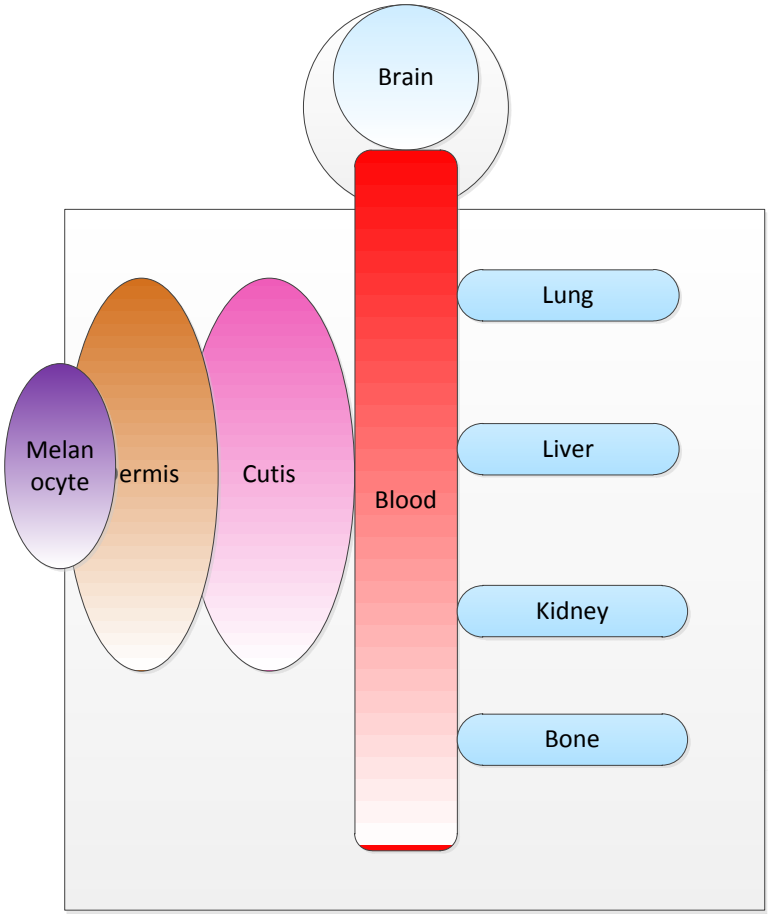
[136] See Andersen p 277 of Bellomo et al for an variant on what we are proposing here. The Andersen model is somewhat similar but lacks the detail we present herein. Also there is in the same volume a paper by Pepper and Lolas focusing on the dynamics of the lymphatic cancer system, p 255.



Here above we describe the three factors in terms of their effects and their causes. The three elements of the equation; diffusion, flow, and growth, are the three ways in which cancer cells move. We can summarize these as below:

Factor	***Diffusion***	***Flow***	***Growth***
Physical Effect	Cancer cells begin to diffuse due to concentration effects.	Cancer cells are "forced" to move by a flow mechanism driven them in a direction along flow lines.	Cancer cells begin to go through mitosis and cell growth.
Genetic Driver	Movement is due to the loss of location restrictors such as E cadherin found in melanocytes and restricting their movement.	Flow lines may be developed by means of metabolic needs of the cell in search of the nutrients required for growth. This may be a combination of angiogenesis as well as a Warburg like effect.	Growth factor ligands attach to the surface of the cell. Flow of such ligands and their production may be influenced by a Turing flow effect thus accounting for complexity of location of growth.
Impact	Slow migration in local areas.	Cells have lost functionality and move to maximize their nutrition input to facilitate growth.	Cancer cells may find optimal areas for proliferation based upon factor related to ligand density.

Now consider the following graphic as a human body,

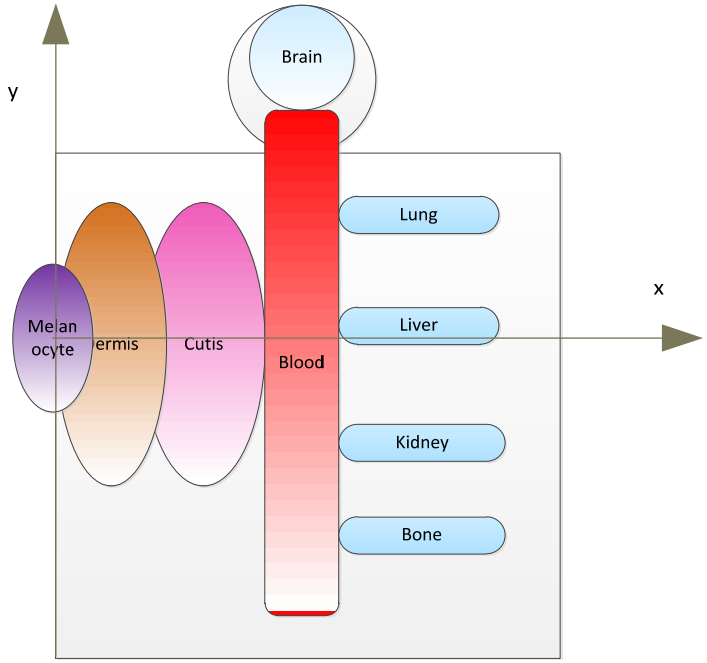


We have a D, E, F, for each gross portion of the body. We also have a model as specifically below in the Table:

Organ	*D* *Diffusion*	*E* *Flow*	*F* *Production*
Epidermis	0.5	0.01	0.7
Dermis	0.4	0.02	0.5
Cutis	0.3	0.05	0.2
Blood	5.0	0.5	0.01
Brain	0.1	0.01	0.2
Liver	2.0	0.2	0.3
Lung	3.0	0.3	0.4
Kidney	1.5	0.4	0.5
Bone	2.5	0.5	1.0

The above numbers are purely speculative. But if we can ascertain them then we get a solution of p(x,t) in time. Note that here we have a two dimensional space. Thus we have the above constants applying only to this artifactually spatial model. Distance is measured in terms of

movement across the interfaces. For simplicity we assume that all other space is impenetrable by any means. This we have production, flow and diffusion in each area.



Note that in the above we have laid out the x and y coordinates such that we have blood flow in the center, namely the metastasis flows via blood, and then enters organs as shown. The "location" of the organs are distances. Note also the origin of the malignancy is at (0,0).

Now we can relate the constants to the pathway distortions which are part of the malignancy as well.

The question is how do we determine these constants so that we may verify the model. Let us assume we can do so via examination of prior malignancy, not an obvious task but one we shall demonstrate. One must be cautious also to include in the determination pathway factors for each malignancy and its state and stage. Thus the three constants will be highly dependent upon the specific genetic makeup of the initial malignancy.

10.4.2 Turing Tessellation

In 1952 Alan Turing, in the last year and a half of his life, was focusing on biological models and moving away from his seminal efforts in encryption and computers. It was Turing who in the Second World War managed to break many of the German codes on Ultra and who also created the paradigm for computers which we use today. In his last efforts before his untimely suicide Turing looked at the problem of patterning in plants and animals. This was done at the same time Watson and Crick were working on the gene and DNA. Turing had no detailed model to work

with, he had no gene, and he had just a gestalt, if you will, to model this issue. Today we have the details of the model to fill in the gaps in the Turing model.

The Turing model was quite simple. It stated that there was some chemical, and a concentration of that chemical, call it C, which was the determinant of a color. Consider the case of a zebra and its hair. If C were above a certain level the hair was black and if below that level the hair was white. As Turing states in the abstract of the paper:

"It is suggested that a system of chemical substances, called morphogens, reacting together and diffusing through a tissue, is adequate to account for the main phenomena of morphogenesis. Such a system, although it may originally be quite homogeneous, may later develop a pattern or structure due to an instability of the homogeneous equilibrium, which is triggered off by random disturbances. Such reaction-diffusion systems are considered in some detail in the case of an isolated ring of cells, a mathematically convenient, though biologically unusual system.

The investigation is chiefly concerned with the onset of instability. It is found that there are six essentially different forms which this may take. In the most interesting form stationary waves appear on the ring. It is suggested that this might account, for instance, for the tentacle patterns on Hydra and for whorled leaves. A system of reactions and diffusion on a sphere is also considered. Such a system appears to account for gastrulation. Another reaction system in two dimensions gives rise to patterns reminiscent of dappling. It is also suggested that stationary waves in two dimensions could account for the phenomena of phyllotaxis.

The purpose of this paper is to discuss a possible mechanism by which the genes of a zygote may determine the anatomical structure of the resulting organism. The theory does not make any new hypotheses; it merely suggests that certain well-known physical laws are sufficient to account for many of the facts. The full understanding of the paper requires a good knowledge of mathematics, some biology, and some elementary chemistry. Since readers cannot be expected to be experts in all of these subjects, a number of elementary facts are explained, which can be found in text-books, but whose omission would make the paper difficult reading."

Now, Turing reasoned that this chemical, what he called the morphogen, could be generated and could flow out to other cells and in from other cells. Thus focusing on one cell he could create a model across space and time to lay out the concentration of this chemical. He simply postulated that the rate of change of this chemical in time was equal to two factors; first the use of the chemical in the cell, such as a catalyst in a reaction or even part of the reaction, and second, the flow in or out of the cell. The following equation is a statement of Turing's observation.

$$\frac{\partial C_1(x,t)}{\partial t} = F_1(C_1, C_2, x, t) + \lambda_1 \nabla^2 C_1(x,t)$$

This is the nonlinear diffusion equation. It allows one to solve for a concentration, C, as a function of time and space. It requires two things. First is the diffusion coefficient to and from cells and second the functional relationship which shows how the chemical is used within a cell.

The question now is how does one link the coefficients in the models. For example if we believe that diffusion D depends on E cadherin concentration, namely as E cadherin decreases then D increases we may postulate:

$$D(x,t) = \kappa(\beta - n_{ECadherin}(x,t))$$

where the constants are to be determined. We know that the more E cadherin the stickier is the cell and the less diffusion that occurs. Thus the above is at the least a first order approximation.

In a similar manner we can relate F to PTEN and p53. We do so as follows:

$$F(x,t) = \kappa_{PTEN}(\beta_{PTEN} - n_{PTEN}(x,t)) + \kappa_{p53}(\beta_{p53} - n_{p53}(x,t))$$

This is merely suppositional. But we do know the following:

1. The genes which are expressed for adhesion and replication are known.

2. We know the pathways for these genes

3. We know the intracellular models controlling these genes.

4. We know that functionally an excess or paucity of a gene has a certain effect.

5. We know that in general in small amounts the world is linear.

6. We know that we can use regression techniques based upon collected data to determine coefficients in a general sense.

Thus we have a fundamental basis to express the following:

$$D(x,t) = \sum_{i=1}^{N} \kappa_i [\beta_i - n_i(x,t)]$$

$$F(x,t) = \sum_{i=1}^{N} \lambda_i [\alpha_i - n_i(x,t)]$$

$$G(x,t) = \sum_{i=1}^{N} \mu_i [\gamma_i - n_i(x,t)]$$

Now we have related intracellular concentrations, which themselves may be temporally and spatially dependent, to the total parameter values for the flow of cells throughout the body. We may also want to relate these to organ specific parameters as well.

Thus what we have achieved is as follows:

1. Model relating intracellular and whole body.

2. Methodology to determine the constants.

3. Methodology to go from patient data to prognostic data.

4. Methodologies to establish possible treatment methodologies. Namely what gene controls will result in what whole body reactions.

10.5 CANCER METABOLISM: AN EXAMPLE OF COMPLEXITY

When discussing the Total Cellular Dynamics model we often encounter the question of where does the cancer cell get its growth energy from? Does it seek paths which offer it the richest environment for expansion or are there other mechanisms that make this unnecessary. We briefly examine the Warburg Hypothesis. Warburg conjectured that cancer is caused by damage to respiration, oxidation, and the cells getting energy via fermentation.

As Warburg noted in 1956:

Cancer cells originate from normal body cells in two phases. The first phase is the irreversible injuring of respiration. Just as there are many remote causes of plague-heat, insects, rats-but only one common cause, the plague bacillus, there are a great many remote causes of cancer-tar, rays, arsenic, pressure, urethane- but there is only one common cause into which all other causes of cancer merge, the irreversible injuring of respiration.

The irreversible injuring of respiration is followed, as the second phase of cancer formation, by a long struggle for existence by the injured cells to maintain their structure, in which a part of the cells perish from lack of energy, while another part succeed in replacing the irretrievably lost respiration energy by fermentation energy. Because of the morphological inferiority of fermentation energy, the highly differentiated body cells are converted by this into undifferentiated cells that grow wildly-the cancer cells

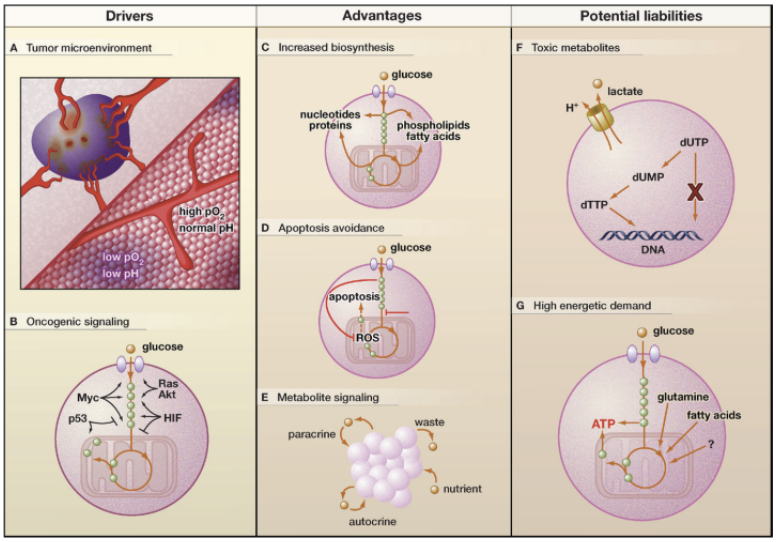
Although the Hypothesis was and is consistent with observations the cause and effect are highly suspect. Yet it does pose the issue of energy infusion in cancer cells.

As Hsu and Sabatini remark concerning Warburg:

It is hard to begin a discussion of cancer cell metabolism without first mentioning Otto Warburg. A pioneer in the study of respiration, Warburg made a striking discovery in the 1920s. He found that, even in the presence of ample oxygen, cancer cells prefer to metabolize glucose by glycolysis, a seeming paradox as glycolysis, when compared to oxidative phosphorylation, is a less efficient pathway for producing ATP (Warburg, 1956). The Warburg effect has since been demonstrated in different types of tumors and the concomitant increase in glucose uptake has been exploited clinically for the detection of tumors by fluoro-deoxy-glucose positron emission tomography (FDG-PET).

Although aerobic glycolysis has now been generally accepted as a metabolic hallmark of cancer, its causal relationship with cancer progression is still unclear. In this Essay, we discuss the possible drivers, advantages, and potential liabilities of the altered metabolism of cancer cells. Although our emphasis on the Warburg effect reflects the focus of the field, we would also like to encourage a broader approach to the study of cancer metabolism that takes into account the contributions of all interconnected small molecule pathways of the cell.

They demonstrate this in the Figure below showing pathway elements and advantages and disadvantages of such signalling:



And Hsu and Sabatini also demonstrate that this pathway control also controls apoptosis:

In addition to involvement in proliferation, altered metabolism may promote another cancer-essential function: the avoidance of apoptosis. Loss of the p53 target TIGAR sensitizes cancer cells to apoptosis, most likely by causing an increase in reactive oxygen species (Bensaad et al., 2006). On the other hand, overexpression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) prevents caspase-independent cell death, presumably by stimulating glycolysis, increasing cellular ATP levels, and promoting autophagy (Colell et al., 2007). Whether or not GAPDH plays a physiological role in the regulation of cell death remains to be determined.

As Vander Heiden and Sabatini state:

In principle, the metabolic dependencies of cancer cells can be exploited for cancer treatment. For instance, a large fraction of human cancer is dependent on aberrant signaling through the PI3K/Akt pathway, and agents that target PI3K and various downstream signaling molecules are now in clinical trials.

The growing evidence that activation of PI3K causes increased dependency on glycolysis suggests that these agents may exert some of their effect by disrupting glucose metabolism. Drugs targeting key metabolic control points important for aerobic glycolysis, such as PK-M2 or

LDH-A, might also warrant investigation as potential cancer therapies. In addition, the drugs developed to target metabolic diseases such as type 2 diabetes may have use in treating cancer. A number of retrospective clinical studies have found that the widely used diabetes drug metformin may offer a possible benefit in cancer prevention as well as improved outcomes when used with other cancer therapies.

Metformin and the more potent related compound Phenformin activate AMPK in cells, suggesting that Phenformin or other activators of AMPK might also be used as an adjunct to cancer therapy. Optimal use of these drugs will require a better understanding of cancer cell metabolism and identification of the signaling pathways that represent an Achilles' heel for cell proliferation or survival.

Metabolic tissues in mammals transform ingested food into a near-constant supply of glucose, glutamine, and lipids to balance the metabolic needs of both differentiated and proliferating tissues. Alterations in the appropriate balance of fuels and/or signal transduction pathways that deal with nutrient utilization may underlie the cancer predisposition associated with metabolic diseases such as diabetes and obesity.

A better understanding of how whole-body metabolism interacts with tumor metabolism may better define these risks and identify potential points of therapeutic intervention. In addition, it is possible that the cachexia associated with many cancers is exacerbated by the excess nutrient consumption by the tumor, which would affect whole-body metabolic regulation.

To this end, the potential role of dietary supplements and tight glucose control as adjuncts to cancer treatment is an active field of investigation.

The last comments has significant merit. The issue of glucose control, the impact of insulin related ligands and the pathway activation, all demonstrate this effect. One may then conjecture that when cancer cells migrate, they may do so along lines of higher glucose concentration. Thus the preference of hematological migration. Again the Warburg Hypothesis is less of interest in the classic sense than in the ability to better understand migratory and diffusional behavior.

10.6 STOCHASTIC MODELS

Stochastic Models are at the heart of understanding how the systems may function under the following circumstances:

1. When truly random factors influence a pathway. There are true random effects. They are akin to white noise or random noise in a radio signal. They are just artifacts of things that have nothing to do with our known system. They may be of a small nature, thus Gaussian in nature, with no big events, or event like, big factors, occurring from time to time, and thus may be Poisson like. Can we measure them, possibly, can we develop a physical model for them, most likely, but for the most part they take no part in the underlying pathway and they are independent totally, and in turn in a statistical manner.

2. Unknown or Known Effects: Let us consider here the miRNA model as an example. We know they exist, we know they disturb pathways, but we do not have a good model for them. Thus we consider them to be random, and we attempt to provide a model based upon measured reality to incorporate their effects. There are positives and negatives with these approaches.

An alternative approach is to include the unknown/known effects as states to be identified, using classic identification techniques. We have employed that approach from time to time but it may suffer from certain estimation instabilities. We shall examine that later.

10.7 STABILITY

With highly complex systems, even just linear ones and more so with nonlinear ones we have significant issues regarding stability. The stability issue may be model related or more interestingly it may reflect the nature of that specific pathway.

The systems we see developed may perforce of their complexity have substantial stability problems. We must be cautious to be assured that the instability if present reflects nature and not the model.

10.8 CONTROLLABLE AND OBSERVABLE

In systems we often are concerned about two key concepts; controllability and observability. Let us first define them and then discuss their importance.

For both cases we assume we have a system of the following type:

$$\frac{dn(t)}{dt} = A(t)n(t) + B(t)u(t)$$
$$z(t) = C(t)n(t)$$

Namely we have some system which has a large vector of proteins in a cell, and that we know the dynamics of these proteins and that we have some control vector u which can control the number of proteins in a cell. Furthermore we assume we can observe the number of proteins via some system which produces a measurement z.

We now introduce the two concepts. We rely upon McGarty (1974, pp 33-41) for the theoretical background

10.8.1 Controllability

Now a system is said to be controllable is we can find some u(t) which allows us to drive the system to some state x(T) and time T. We may not find the specific u immediately but we are assured that one exists.

This is a critical concept since it allows us to say develop a protocol to treat some pathway disorder by selecting some control u, say a selection of a kinase inhibitor. On the other hand if

the system is not controllable that negative result may force us to expand the system or try and find an alternative set of controls by changing say the C matrix.

10.8.2 Observability

A system is observable if from a set of measurements, z(t), where tε(r,s), we can determine x(T) for some T. Namely we assume we know the dynamics of the system and that we have measurements over some interval and from these measurements we can then determine x(T) and in turn any other value of x since we know the governing equations. In our case at hand, we assume we have say some microarray or FISH measurements, or others, and that from these we can then determine all of the protein counts at some T and thus at any t. Namely the system will be fully determined.

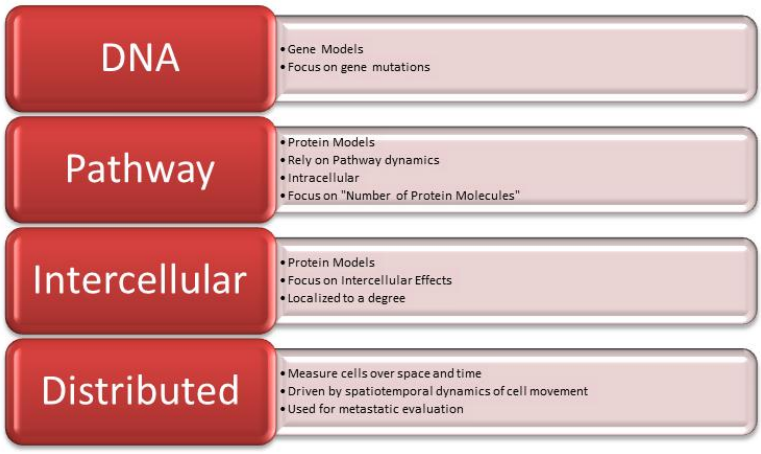
Thus with these two concepts we have a model for a system which can be used to both identify all states and drive it to a desired state.

10.9 SUMMARY

We can now summarize this models we have considered. First we should emphasize that for the most part those working in the field have developed pathway models which exhibit a non-temporal mode, it is some steady state model, and the model assumes a protein to protein connection, as if there were a single protein molecule produced and that the interacting proteins were there or not. Part of the simplicity of the models is determined by the limits of what can be measured. We have herein attempted not to limit the results by what can be accomplished currently but has extended the model to levels which assist in a fuller representation of reality. However even here we may very be falling short.

For we have deliberately neglected such things as miRNA, methylation, and the stem cell paradigm just to name a few.

We combine all four methods in a graphic below. We summarize the key differences and differentiators. Currently most of the analytical models focus on pathways. This can generally be supported by means of microarray technology and even rough estimates of relative concentrations may be inferred by such an approach.



The risks we see even in the above models is the absence of exogenous epigenetic factors and the inclusion of a stem cell model. The latter issue is one of major concern. For example if we have true cancer stem cells, CSC, then we have a proliferation of differing cell types. The use of microarrays is for the most part and averaging methodology, not a cell by cell methodology. If we collect cells from say a melanoma tumor. how much of that is a CSC and how much a TIC. And frankly should we identify CSCs only and perform our analysis on those cells alone.

10.10 Mutations and Protein Expression

We have previously introduced a cancer cell propagation model in earlier discussions which others have also considered. However our model is for a single cell type which proliferates diffuses and flows. The rates of each are dependent on where the cell is. Namely the rates of proliferation, diffusion, and flow depend on the external environmental factors. Thus we often find that proliferation may be enhanced when a cancer cell has metastasized to the liver or brain. The cancer cell may just flow and not proliferate in the blood stream. And the cancer cell may just diffuse at it moves through the vascular wall. The environment is a key determinant.

We now add other elements, namely the probability that a cell can mutate and that as it mutates the factors related to the propagation model may also change. We know that cancer cells do not just have one mutation, but a process of such mutations. The cell may start with a specific change, such as loss of E cadherin for a melanocyte which then allows the cell to move from the basal layer. This may result in a melanoma in situ.

Then we get a mutation in BRAF which allows for proliferation of the movable melanocytes and then loss of p53 for example. Thus there may be a progression of genetic or epigenetic changes in the cell. We now develop a Markov model for this progression, and then we identify collections of cells with the same mutations and apply the same proliferation, flow, and diffusion to each. We calculate a similar diffusion equation now for the average number of malignant cells by region and by type.

10.10.1Added Complexity

The previous model described a single mutation. This expands the model by addressing multiple mutations in a Markov manner. That is we demonstrate:

1. The standard diffusion-flow-proliferation model applies on a per-region and per cell type basis. This means that the constants we have developed previously will depend on the specific cell type as well, namely how many mutations have occurred.

2. That we know there are multiple mutations in cancer cells. Some may have a few and are indolent and others may have many and be aggressive. We develop a Markov model for such cell progression.

3. We combine the three element spatio-temporal model with the Markov cell mutation model and this allows us to determine the average number of cells of a specific type in any part of the body at any point in time.

4. We then discuss how one may use this model for prognostic and therapeutic purposes.

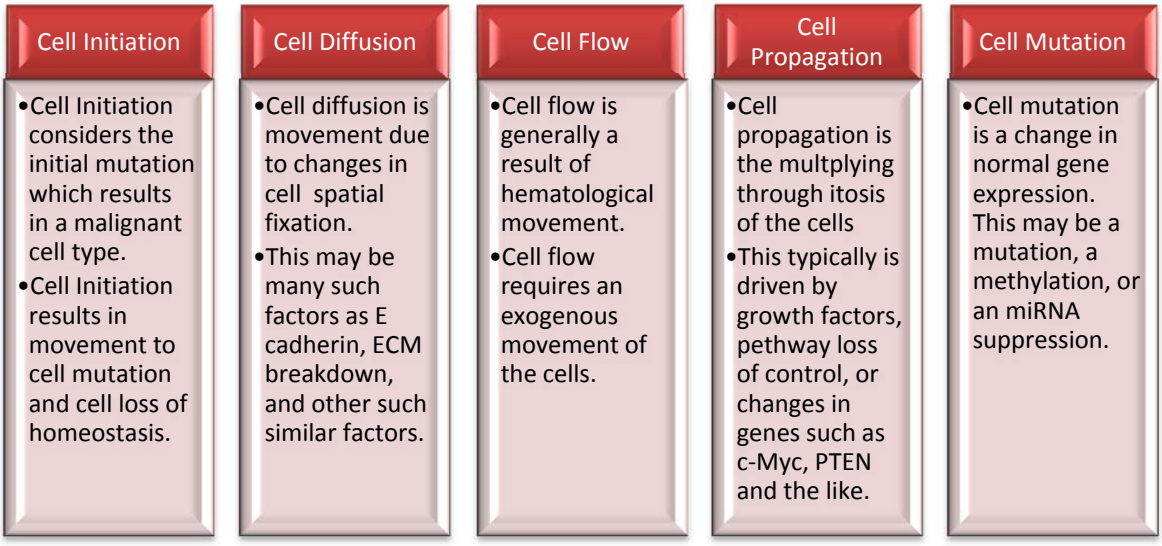
The main observation in this brief section is that the average number of malignant cells of a specific mutation state can be determined by the following equation:

$$\frac{\partial \overline{n(x,t)}}{\partial t} = \tilde{L}\overline{n(x,t)} + \Lambda \overline{n(x,t)}$$

In this equation the n is an NX1 vector of average numbers in spatio-temporal dependent values of each of N possible mutations and the L value is the spatio-temporal dependent operator matrix and Λ is a matrix describing the Markov transition probabilities between mutations.

It should be clear that we can measure all of the constants involved and thus determine the result. As a counter-distinction we can measure the n values and mutation states and determine the constants.

The expanded model considers the issue diagrammed below:



The next issue is the ability to determine what the factors are in the specific model, namely the values of the constants, and secondly the validation of the model itself.

10.10.2Key Issues

Thus there are two dimensions of key issues here:

1. Model Identification and Validation: In previous work we referred to this as the Observability problem. Namely if we have a model and we can identify the required parameters, then can this model be used to determine the end state which will be attained. This is the prognostic problem.

2. Model Utilization: As with the previous cases, if we have this model, and we have identified the constants, can we determine actions which may be taken to control the end state of the system? This is the Controllability problem. It states that perhaps having such a model we can determine methods and means to drive the system, in this case the average number of malignant cells of genotype say G, to a new end state, one where we have reduced the number of bad cells to a de minimis level. This is the therapeutic problem.

There also is a third element:

3. Identification: In both of the two previous issues we assumed that there existed a method by which we could determine the constants of diffusion et al and furthermore that we could ascertain the list of possible mutations, and also their Markov transition probabilities. This may be accomplished in two ways. First, we can accomplish this by in vitro studies. Second, we can achieve this by using the model itself in a classic system identification model with in vivo analyses.

Thus the analysis contained herein is an initiation of what appears to be an innovative way to look at cancer. There have been many studies in more specific and segmented areas but there has not to my knowledge been a study that has examined cancer in such a broad and overarching manner. In essence we have included all of the variables that one may ask for.

10.10.3Understanding Metastasis

This model is one which attempts to understand metastasis from two dimensions. First, we have examined the movement of malignant cells around the body. The movement and proliferation is driven by the cell dynamics such as the mitotic cycle and its control and the loss of cell specificity and spatial stability.

Metastasis is a somewhat unique characteristic of cancer. Viral diseases, such as HPV and similar viruses, which cause warts, are highly localized. They cause proliferation but localization is maintained.

Finally, recall that with metastasis we have the following typical sites[137]:

Cancer type	Main sites of metastasis*
Bladder	Bone, liver, lung
Breast	Bone, brain, liver, lung
Colorectal	Liver, lung, peritoneum
Kidney	Adrenal gland, bone, brain, liver, lung
Lung	Adrenal gland, bone, brain, liver, other lung
Melanoma	Bone, brain, liver, lung, skin/muscle
Ovary	Liver, lung, peritoneum
Pancreas	Liver, lung, peritoneum
Prostate	Adrenal gland, bone, liver, lung
Stomach	Liver, lung, peritoneum
Thyroid	Bone, liver, lung
Uterus	Bone, liver, lung, peritoneum, vagina

Thus for each of the above sites we have a local cellular and extracellular environment which supports the metastatic behavior that we often see in such cells.

Finally we discuss some of the issue of how do we ascertain the constants in each of the models and this includes the Markov transition probabilities. We examine several approaches, invasive and non-invasive ones. We believe that molecular functional imaging, MFI, provides an attractive approach to ascertaining these constants.

137 http://www.cancer.gov/cancertopics/factsheet/Sites-Types/metastatic?utm_source=feedburner&utm_medium=feed&utm_campaign=Feed%3A+ncifactsheets+%28NCI+Fact+Sheets%29

10.11 SINGLE CELLULAR TYPE

We have previously developed a simple model for the change in the number of cells of a specific type at a specific place and time as follows.

10.11.1The Model

We have demonstrated earlier that for a specific type of single mutated cell that the number of such malignant cells at a specific time t and place x are determined by n(x,t) and that this can be described by the following equation.

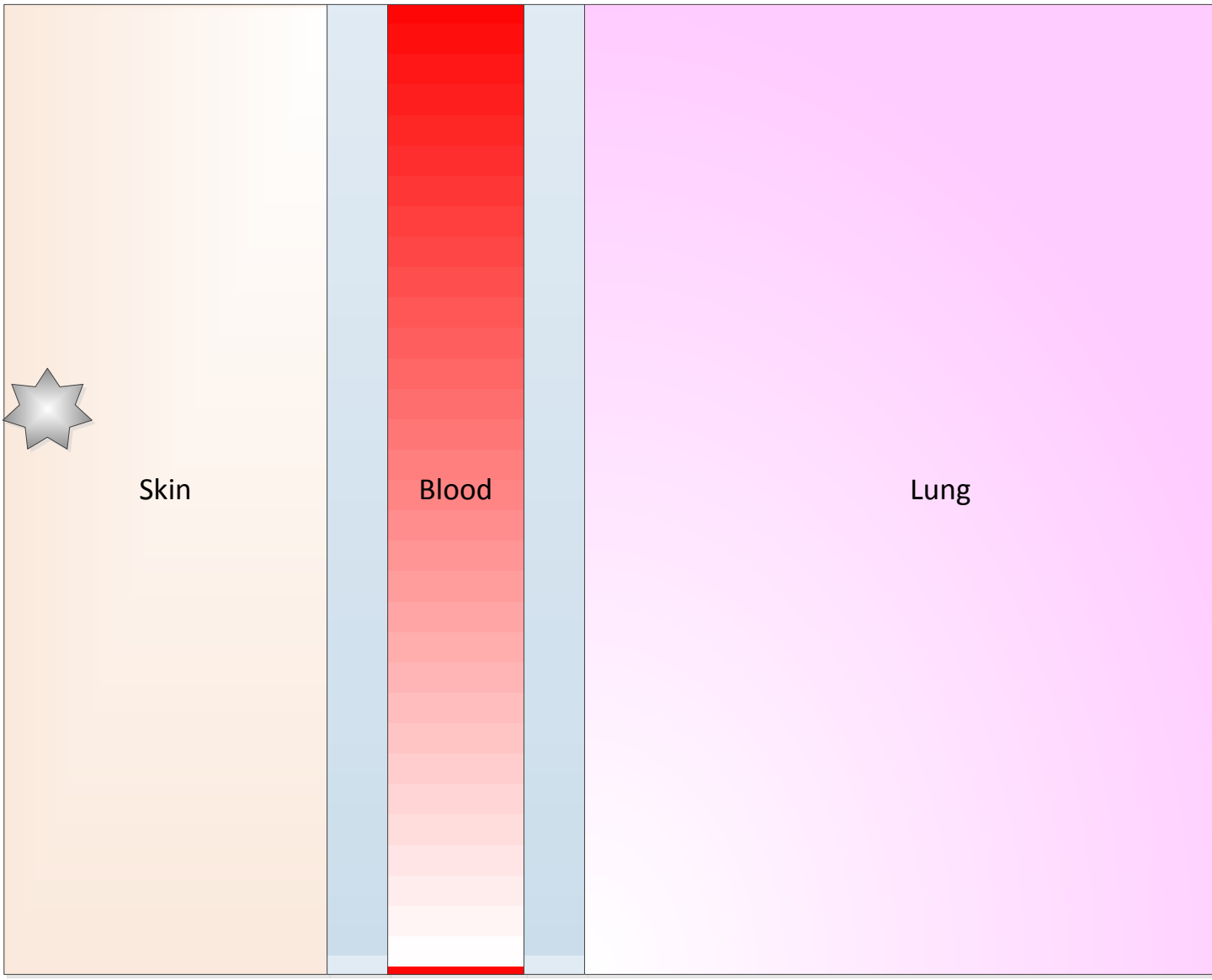
$$\frac{\partial n(x,t)}{\partial t} = a\frac{\partial^2 n(x,t)}{\partial x^2} + b\frac{\partial n(x,t)}{\partial x} + cn(x,t)$$

This depicts; diffusion, flow, and growth. The coefficients are cell type dependent and may also be spatially and temporally dependent also. We need not worry about that at this time.

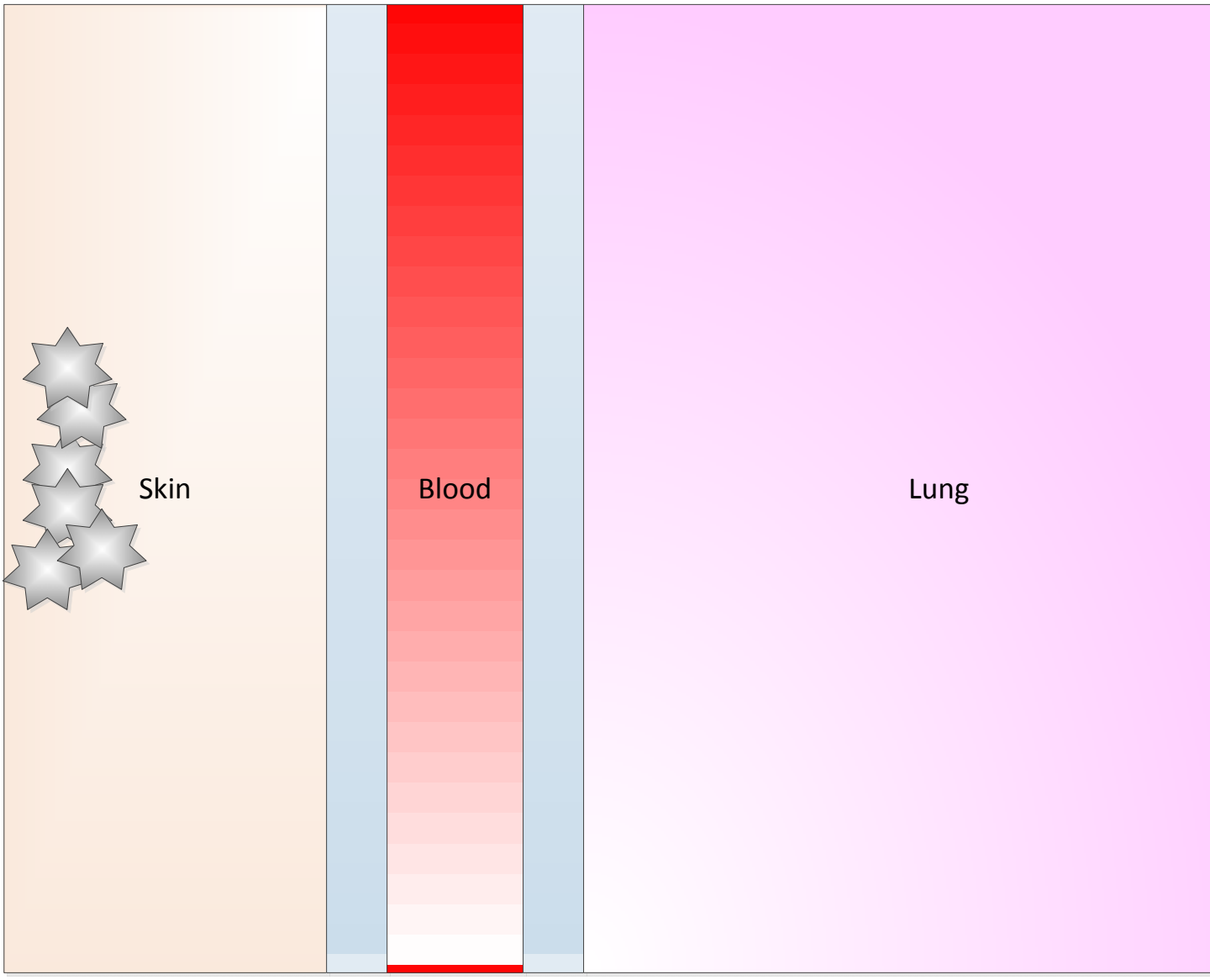
10.11.2An Example

To better understand we depict the progression of melanoma below with a simple graphical example. We start with a simple benign cell, assume a single malignant change and then follow the proliferation and movement of the cell. This graphic makes many simplified assumptions which we shall release shortly..

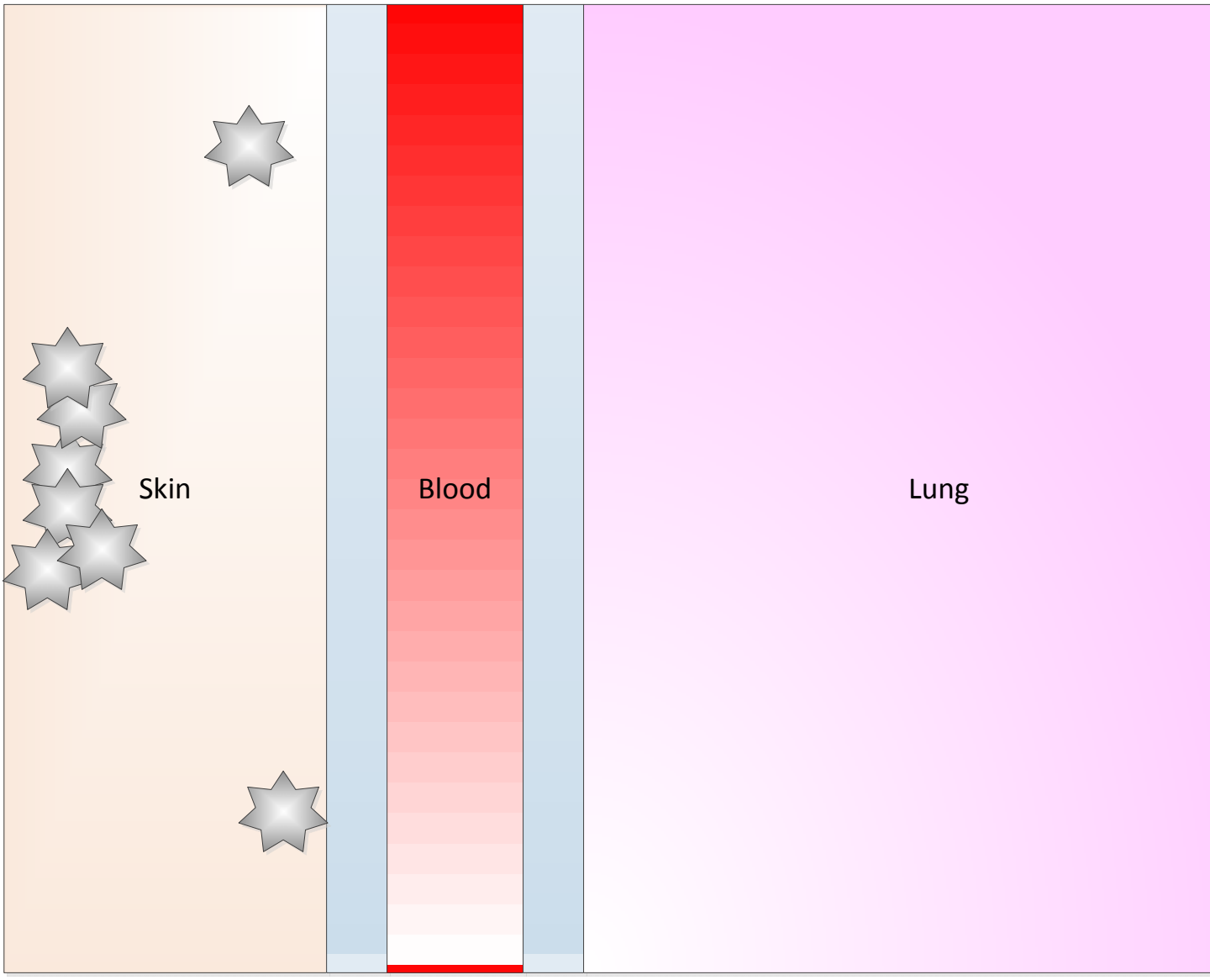
Step 1: Benign State, here we have five segments; skin, two tissue-blood barriers, blood, and lung. We begin by showing a single melanocyte. We assume the melanocyte is affixed to the basal layer with E cadherin functioning properly.



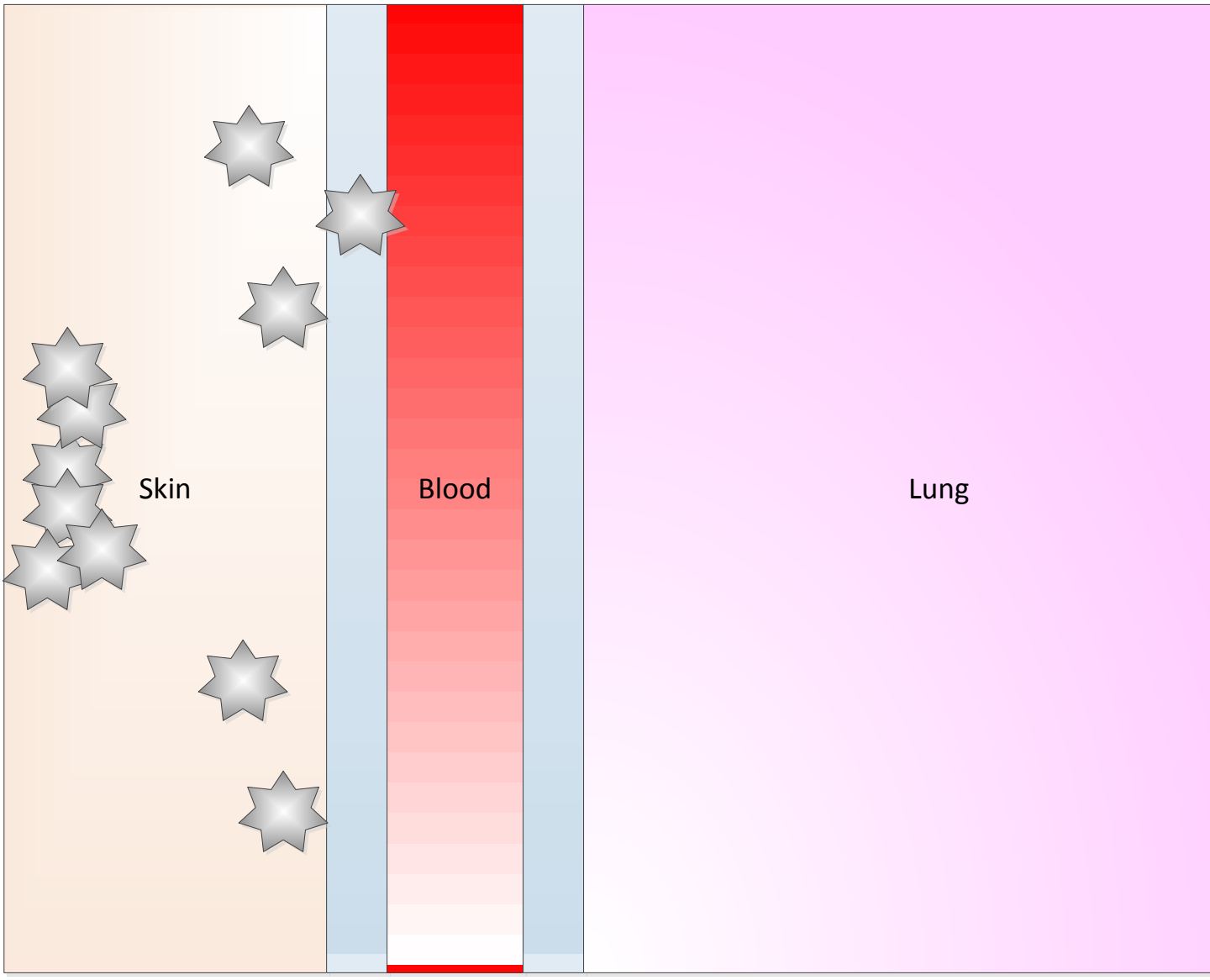
Step 2: We have the beginning of a cancer due to some mutation of the basal or luminal cells. The cancer initially proliferates and then it diffuses. In the figure below we show that it is still localized.



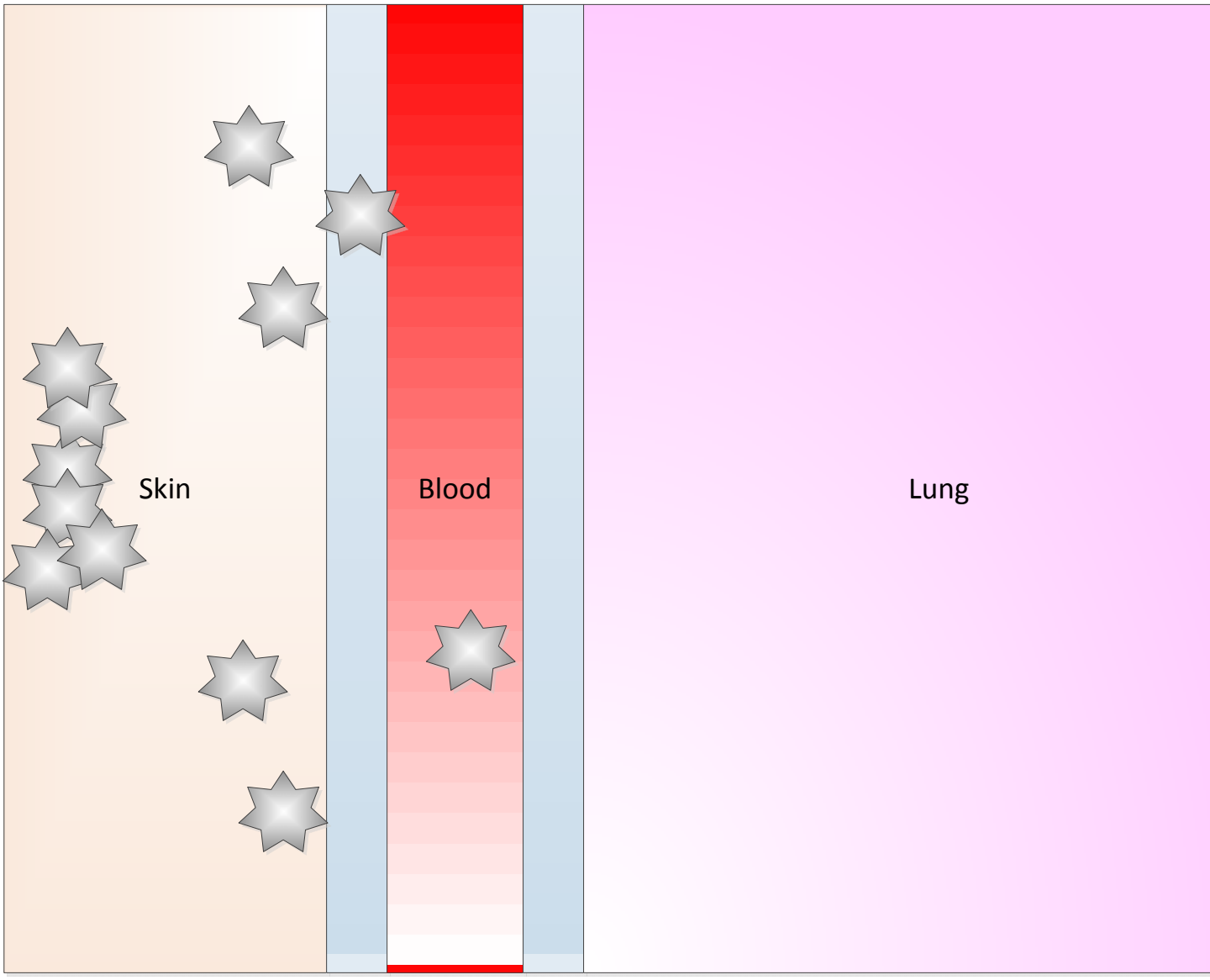
Step 3: Diffusions begins and starts to send the cancer cells towards the blood barrier.



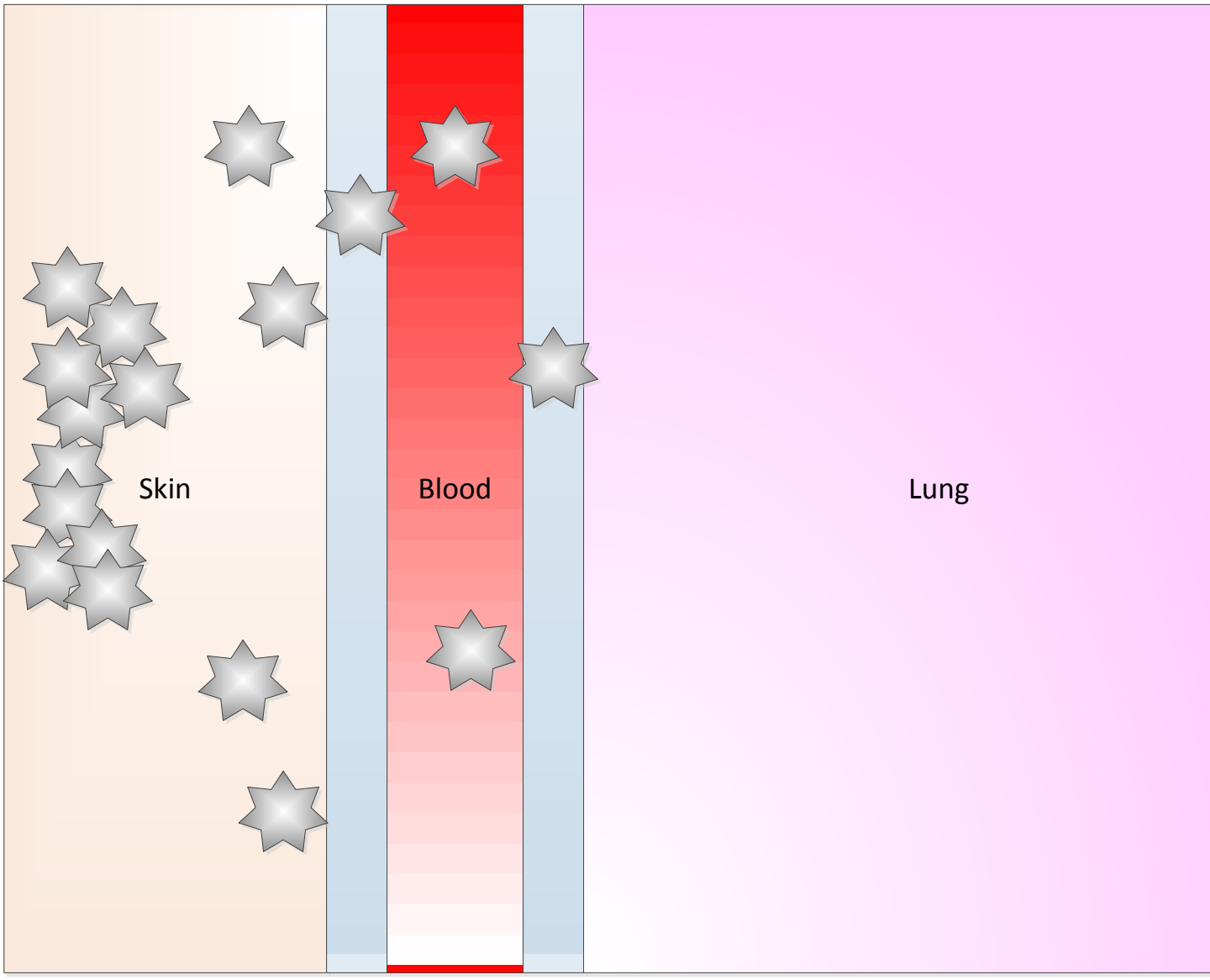
Step 4: The blood barrier is crossed, and we assume by diffusion. Across this barrier there is no proliferation or flow, just diffusion.



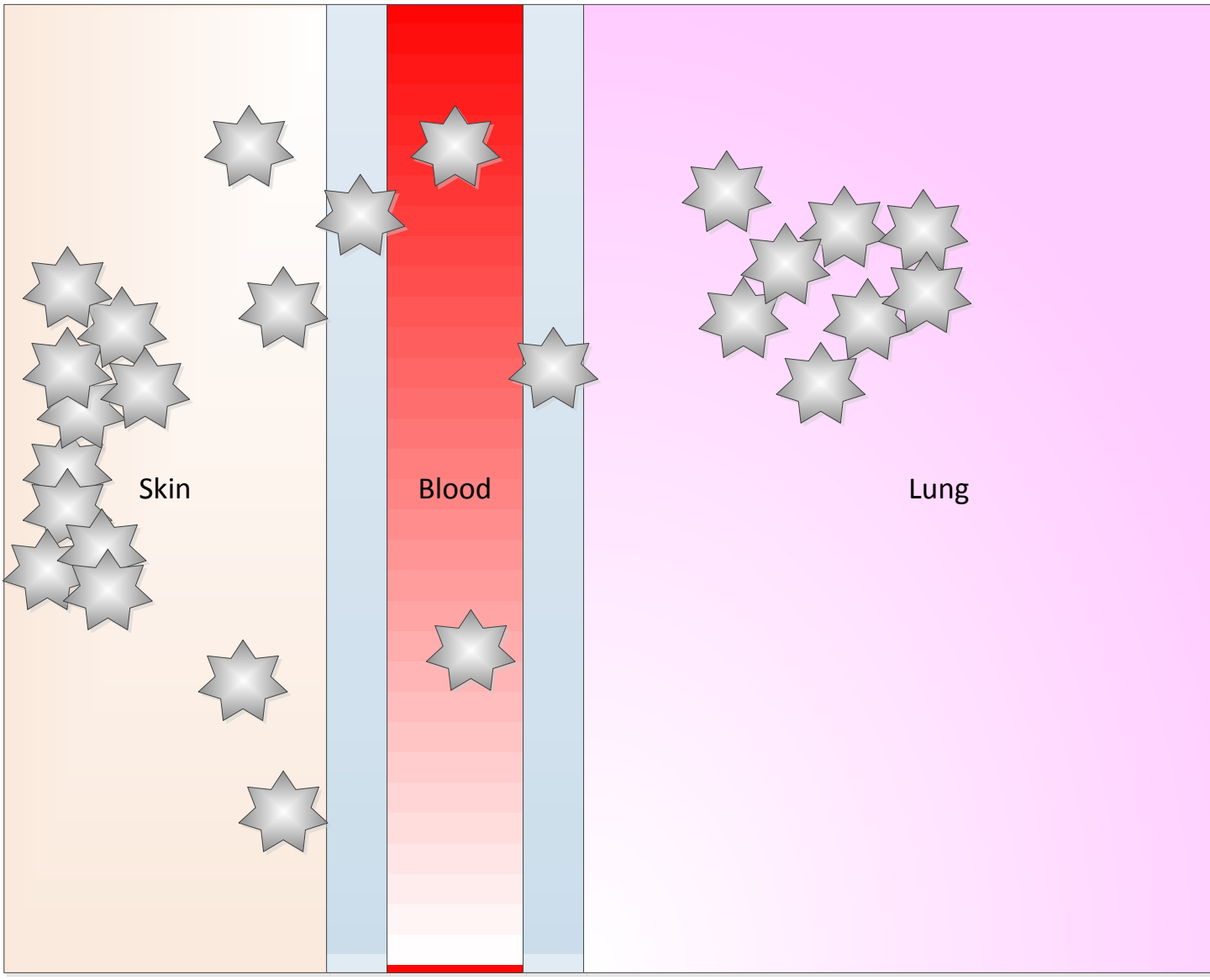
Step 5: The blood barrier is crossed and the cell is now in the blood stream. Here we have flow but no diffusion and no proliferation.



Step 6: The blood barrier is crossed again as discussed above.



Step 7: Metastasis is complete by having the new malignant cells in the lung and proliferation and diffusion predominate.



The above steps are common is almost all cancers. The assumptions here are:

1. The same malignant cell moves across the body.

2. Each separate area, in this case five, has constant diffusion, flow and proliferation constants.

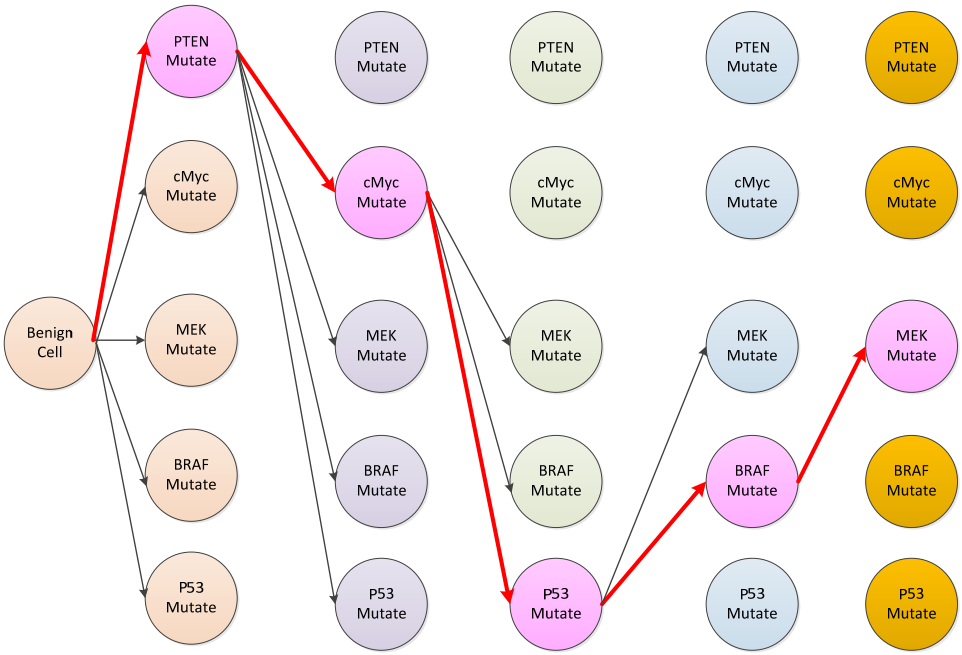
3. That we can then measure the number of cells from this deterministic model.

In the case where they are uniform constants we can solve the equation. In the case where they are uniform constants across uniform spatial domains then we can also solve the equations evoking boundary conditions.

We now want to expand this model to include multiple malignant cell types. Also we want to include their stochastic dynamics as well.

10.12 MARKOV MODELS

Consider a cell with five possible mutations. We show the genes below. The call may begin with one mutation and then move to a second and so forth. Each path is assumed to be possible and the results of each path are different.



Now we can consider a model for the above simple example. We have 5 possible mutations and they may occur in any order. We assume they occur one at a time. We can identify any number of cells as:

$$n_{1,k}(x,t)$$

As the number of cells after one mutation at location x and at time t, of mutation k.

10.12.1The Markov Assumptions

Now we have the following observations:

1. At mutation 1 we have 5 possible cell mutants. Furthermore each may be considered a cancer cell and the growth, diffusion and flow are as described above. Some of the mutations may be indolent and some aggressive.

2. At mutation 2 we have 5*4 possible cells. The question is that some are say PTEN then cMyc or cMyc then PTEN. Are they the same, and this means the difference between perturbation and

combination? Are they distinct by have been ordered differently or are they the same? If it is a combination we have 10 instead of 20 different mutations.

3. At mutation 3 we have 5*4*3 and at 4 we have 5*4*3*2 or 120 permutations.

4. At any location we may have any one or a combination of these mutation types. There are two factors driving their number:

a. A single type will have growth, dispersion and movement dynamics with the above mentioned model but each mutation will respond differently since their coefficients will be different. Some may grow faster and some may diffuse faster. There is no a priori ranking of the coefficients.

b. The surrounding mutant types will also tend to mitigate growth.

Now we can call the mutant cells as follows:

$n_{1,k}(x,t)$
$n_{2,j,k}(x,t)$
$n_{3,i,j,k}(x,t)$
$n_{4,h,i,j,k}(x,t)$
$n_{5,g,h,i,j,k}(x,t)$

Now we know how the subscripts can be ordered as per the above conversation. But we also assume that there exist some Markov mode for transitions from a 1 state to a 2 state, namely from one mutation to 2 mutations. That is we can assume a discrete time discrete state system and ascribe a Markov model with transition probabilities. Namely:

$$P\left[x(k+1) = x_0 \mid x(k) = x_0\right] = p_0$$
$$P\left[x(k+1) = x_{1,k} \mid x(k) = x_0\right] = p_{k,0}$$

Where we have the standard closure conditions on the transition probabilities. The process is Markov and it depends solely upon the prior state and no other.

In general we have:

$$P\left[x(k+1) = x_{n,k} \mid x(k) = x_{n,k}\right] = p_{n,k}$$
$$P\left[x(k+1) = x_{n+1,m} \mid x(k) = x_{n,k}\right] = p_{n:m,k}$$

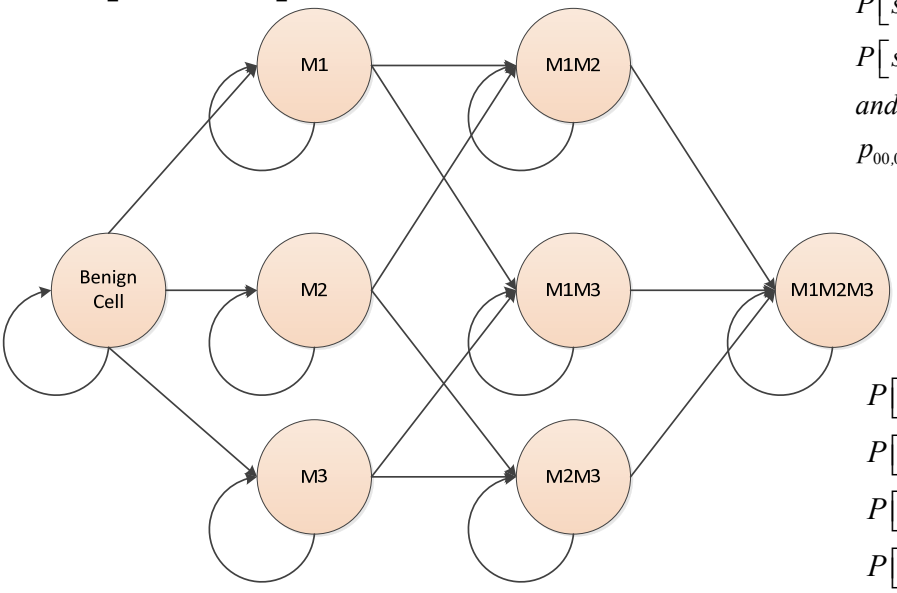
We can extend this to a continuous time system simply. We just need the mutation rates.

Now the initial equation describing single mutant growth was deterministic. However we now have demonstrated a random process. Thus we want to determine the average number at any time and at any location. The average will include the temporal Markovian dynamics of cell mutation rates which themselves may be spatially dependent.

10.12.2The Markov Example

For example consider the following three gene mutation case:

$$P = \begin{bmatrix} p_{11} \cdots p_{21} \cdots p_{81} \\ \cdots \\ p_{18} \cdots p_{28} \cdots p_{88} \end{bmatrix}$$



$$P\left[s00 \middle| s00\right] = p_{00,00}$$
$$P\left[s11 \middle| s00\right] = p_{11,00}$$
$$P\left[s12 \middle| s00\right] = p_{12,00}$$
$$P\left[s13 \middle| s00\right] = p_{13,00}$$
and
$$p_{00,00} + p_{11,00} + p_{12,00} + p_{13,00} = 1$$

$$P\left[s11 \middle| s11\right] = p_{11,11}$$
$$P\left[s21 \middle| s11\right] = p_{21,11}$$
$$P\left[s22 \middle| s11\right] = p_{22,11}$$
$$P\left[s23 \middle| s11\right] = p_{23,11}$$
and
$$p_{11,11} + p_{21,11} + p_{22,11} + p_{23,11} = 1$$

Now for this simple example we can assume that 1 is the start and 8 is the end. We also assume that 2, 3, 4 are the first steps and 5, 6, 7 are the second. Then we have a transition probability matrix P as follows:

$$P = \begin{bmatrix} p_{1,1}..p_{2,1}..p_{2,2}..p_{2,3}..0.....0.....0.....0 \\ 0.....p_{2,2}..p_{2,5}..p_{2,6}..p_{2.7}..0.....0.....0 \\ 0.....0.....p_{3,3}..p_{3,5}..p_{3,6}..p_{3,7}..0.....0 \\ 0.....0....0.....p_{4,4}..p_{4,5}..p_{4,6}..p_{4,7}.....0 \\ 0.....0....0.....0.....p_{5,5}..0.....0.....p_{5,8} \\ 0.....0....0.....0.....0.....p_{6,8}.....0.....p_{6,8} \\ 0.....0....0.....0.....0.....0.....p_{7,8}....p_{7,8} \\ 0.....0....0.....0.....0.....0.....0.....p_{8,8} \end{bmatrix}$$

Where the sum across any row is unity. Thus we have:

$$p(k+1) = Pp(k)$$

where

$$p(k) = \begin{bmatrix} p_1(k) \\ ... \\ p_8(k) \end{bmatrix}$$

In this specific example.

Where p is the probability vector of all possible states, say 8 in our previous example, and P is the transition probability matrix. Note we have assigned P as dependent upon x and t. The same holds true for p.

Let us consider a simple example of N possible states and we examine the probability density of a specific state, Let:

$$p(n_i; x.t)$$

Where p is the probability density function of the random variable n on gene mix i. We assume that n is a continuous random variable so we can provide a simpler representation.

Now we can move to a continuous time simply by stating:

$$\frac{dp(x,t)}{dt} = P(x,t)p(x,t)$$

However we can simplify this as follows:

$$\frac{dp(n_i;x,t)}{dt} = -\lambda_{ii}p(n_i;x,t) + \sum_{j=1,j\neq i}^{N} \lambda_{i.j}p(n_j;x,t)$$

This is the continuous version of the above and is a simple Markov birth-death type model.

Now consider any point x, t in space-time. We can define:

$$E\left[n_i(x,t)\right] = \int n_i(x,t)p(n_i;x,t)dn_i$$
$$= \overline{n_i(x,t)}$$

We shall use this in the next section.

10.13 COMBINING

We now will combine the propagation equation model for a single malignant state with the overall evolution of malignant states via a Markov model.

Now consider a specific cell count at some specific x and t. We know we have:

$$\frac{\partial n_k(x,t)}{\partial t} = Ln_k(x,t)$$

Where L is the spatial operator.

We also have for the probability of each n being a specific value at a specific x and t as:

$$\frac{\partial p_k(x,t)}{\partial t} = \sum_{n=1}^{N} \lambda_n(x,t) p_n(x,t)$$

Now we can define the average of any n as follows:

$$\overline{n_k(x,t)} = \sum p_k(n_k(x,t) = n_m) n_m(x,t)$$

This is the average value of that specific n value for that specific gene mutation at that specific time and location. We have a means to calculate each of the probabilities and we then just calculate the average.

Now we can combine the equations as follows:

$$\sum p_k(x,t) \frac{\partial n_k(x,t)}{\partial t} = \sum \lambda_m(x,t) p_m(x,t) Ln_k(x,t)$$

or

$$\frac{\partial \sum p_k n_k}{\partial t} = L \sum \lambda_{m,k}(x,t) p_{m,k}(x,t) n_k(x,t)$$

or

$$\frac{\partial \overline{n_k(x,t)}}{\partial t} = L \lambda_k \overline{n_k(x,t)}$$

Let me redo the notation again. We will assume that n can be continuous for notation purposes. Thus we have:

$$\overline{n_k(x,t)} = \int p_k(n_k; x,t) n_k(x,t) dn_k$$

Now let us remember that for N possible n types we have:

$$\frac{dp_k(u_k;x,t)}{dt} = \sum_{j=1}^{N} \lambda_{k,j} p_j(u_j;x,t)$$

We have N equations like this.

If we multiply the propagation equation for any n as follows:

$$\frac{p_k(n_k;x,t)\partial n_k(x,t)}{\partial t} = p_k(n_k;x,t)Ln_k(x,t)$$

Now introduce the integral:

$$\int p_k(n_k;x,t)\frac{\partial n_k(x,t)}{\partial t} dn_k = \int p_k(n_k;x,t)Ln_k(x,t)dn_k$$

But recall that we can write:

$$\frac{\partial pn}{\partial t} = p\frac{\partial n}{\partial t} + n\frac{\partial p}{\partial t}$$

Then using what we know of p we have:

$$\frac{\partial p_k(n_k;x,t)}{\partial t} = -\lambda_{k,k} p_k(n_k;x,t) + \sum_{j=1;j\neq k}^{N} \lambda_{k,j} p_j(n_j;x,t)$$

Substituting and rearranging:

$$p_k(n_k;x,t)\frac{\partial n_k(x,t)}{\partial t} = \frac{\partial[p_k(n_k;x,t)n_k(x,t)]}{\partial t} - n_k(x,t)\frac{\partial p_k(n_k;x,t)}{\partial t}$$
$$= \frac{\partial[p_k(n_k;x,t)n_k(x,t)]}{\partial t} - n_k(x,t)\lambda_{k,k} p_k(n_k;x,t) + n_k(x,t)\sum_{j=1;j\neq k}^{N} \lambda_{k,j} p_j(n_j;x,t)$$

Now we can show that when we integrate to obtain averages we have:

$$\int p_k(n_k;x,t)\frac{\partial n_k(x,t)}{\partial t} dn_k = \frac{\partial \overline{n_k(x,t)}}{\partial t} - \lambda_{k,k}\overline{n_k(x,t)} - \sum_{j=1;j\neq k}^{N} \lambda_{k.j} n_j(x,t)$$

Thus we can replace this in the propagation equation to read:

$$\frac{\partial \overline{n_k(x,t)}}{\partial t} - \lambda_{k,k}\overline{n_k(x,t)} - \sum_{j=1;j\neq k}^{N} \lambda_{k.j} n_j(x,t) = L\overline{n_k(x,t)}$$

Now if we had multiplied by the joint pdf we would have obtained:

$$\frac{\partial \overline{n_k(x,t)}}{\partial t} - \lambda_{k,k}\overline{n_k(x,t)} - \sum_{j=1;j\neq k}^{N} \lambda_{k.j} \overline{n_j(x,t)} = L\overline{n_k(x,t)}$$

Or; upon rearranging we obtain:

$$\frac{\partial \overline{n_k(x,t)}}{\partial t} = L_k\overline{n_k(x,t)} - \lambda_{k,k}\overline{n_k(x,t)} + \sum_{j=1;j\neq k}^{N} \lambda_{k.j} \overline{n_j(x,t)}$$

Thus the result for the average is a set of linked partial differential equations. Note we have modified the L operator to reflect specificity for k. The added terms reflect the movement of cell types from one class to another.

This is a powerful equation. It tells us how specific cells diffuse, flow and reproduce, and then how they migrate to new types of cells.

Let us take it one step further. Recall:

$$L_k = a_k \frac{\partial^2}{\partial x^2} + b_k \frac{\partial}{\partial x} + c_k$$

define

$$\widetilde{L_k} = L_k - \lambda_{k,k}$$

Now consider a vector of all n possibilities and we can determine the average vector of these as follows:

$$n(x,t) = \begin{bmatrix} n_1(x,t) \\ \dots \\ n_N(x,t) \end{bmatrix}$$

And where the average of the vector is the average of the above. Then we readily have the equation for all n as follows:

$$\boxed{\frac{\partial \overline{n(x,t)}}{\partial t} = \tilde{L}\overline{n(x,t)} + \Lambda\overline{n(x,t)}}$$

Where:

$$\tilde{L} = \left[\tilde{L}_1 ..... \widetilde{L_N} \right]$$

and

$$\Lambda = \begin{pmatrix} -\lambda_{11} & \lambda_{12} & \lambda_{13} \\ \lambda_{21} & -\lambda_{22} & \lambda_{23} \\ \lambda_{31} & \lambda_{32} & -\lambda_{33} \end{pmatrix}$$

The above is suggestive and it depends on the specific model.

10.14 OBSERVATIONS

To summarize the following depicts the major analytical results:

Propagation Model: This equation provides a spatio-temporal model for the calculation of the number of specific cancer cells which are propagated by means of: (i) diffusion, (ii) flow, and (iii) proliferation.

$$\frac{\partial n(x,t)}{\partial t} = a\frac{\partial^2 n(x,t)}{\partial x^2} + b\frac{\partial n(x,t)}{\partial x} + cn(x,t)$$

Average Model: This model considers the calculation of the average number of malignant cells in a spatio-temporal manner when the cells mutate into N possible genetic variants. It calculates the average number by variant and thus is a vector equation containing the N variants.

$$\frac{\partial \overline{n(x,t)}}{\partial t} = \tilde{L}\overline{n(x,t)} + \Lambda\overline{n(x,t)}$$

We can thus make several important observations regarding this model.

1. Prognostic and Therapeutic: We can determine the transitions and the factors related to diffusion, flow and growth. Thus we can use the result as a powerful one for prognostic and therapeutic results. As we had indicated earlier, the Observability and Controllability issues are essentially Prognostic and Therapeutic respectively.

2. Variances: The results are for the average. We can determine the results for the variances as well. We have examined the variances on the averages and they are somewhat complex and we do not believe that they lend significant additional information at this time.

3. Solutions: The solutions to these equations are readily obtained using standard techniques. They can, in addition, be determined in closed form results.

10.14.1 Measuring the Parameters

Measuring the parameters in these models has been discussed before for a single mutation. However, we have now introduced a set of multiple and progressive mutations. How do we know how these mutations progress? Can the mutation progressions be determined a priori or do they occur in some random fashion? How do we deal with the epigenetic elements such as hypermethylation and miRNAs when we consider changes in expression without mutations?

There are a significant number of questions that we must consider when examining the change in gene expression. In addition the environment, extracellular matrix as well as surrounding cells may also effect changes in gene expression.

Thus ascertaining gene mutation or expression would most likely be determined by examining the cells expression themselves. At the current time there does not appear to be a robust theory which can be used as a basis for such projections. We are left measuring what has actually happened rather than projecting what will occur.

Perhaps subsequent Bayesian analysis will allow for such determination.

10.14.2In Situ Hematological Measurements

The challenge is determining of a cancer has metastasized is to find out where and how much. The classic approach is to look at the local draining lymph nodes and see if has gone there. However the cancer cells may often escape through the blood system and not the lymph system. Consider ocular melanoma, there is no lymph system connection and it spreads by hematological means only.

That means that by examining the blood we should be able to find the wandering malignant cells, at least in theory. In a recent release by *MedGadget* the article relates developments at MGH in Boston as follows[138]:

Circulating tumor cells (CTCs) are shed by primary tumors and allow the cancer to metastasize to the distant sites. While this is a devastating tool in cancer's war chest, it offers clinicians a marker through which to diagnose and monitor progress of the disease. Since the discovery of CTCs over a hundred years ago, researchers have been developing ever more sensitive methods of capturing them since they're extremely rare in whole blood.

In a recent development by Ozkumur et al at MGH[139] the authors' state:

Circulating tumor cells (CTCs) are shed into the bloodstream from primary and metastatic tumor deposits. Their isolation and analysis hold great promise for the early detection of invasive cancer and the management of advanced disease, but technological hurdles have limited their broad clinical utility. We describe an inertial focusing–enhanced microfluidic CTC capture platform, termed "CTC-iChip," that is capable of sorting rare CTCs from whole blood at 107 cells/s.

Most importantly, the iChip is capable of isolating CTCs using strategies that are either dependent or independent of tumor membrane epitopes, and thus applicable to virtually all

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[138] http://www.medgadget.com/2013/04/mgh-ctc-ichip-sets-new-bar-for-circulating-tumor-cell-detection.html

[139] http://stm.sciencemag.org/content/5/179/179ra47

cancers. We specifically demonstrate the use of the iChip in an expanded set of both epithelial and nonepithelial cancers including lung, prostate, pancreas, breast, and melanoma.

The sorting of CTCs as unfixed cells in solution allows for the application of high-quality clinically standardized morphological and immunohistochemical analyses, as well as RNA-based single-cell molecular characterization. The combination of an unbiased, broadly applicable, high-throughput, and automatable rare cell sorting technology with generally accepted molecular assays and cytology standards will enable the integration of CTC-based diagnostics into the clinical management of cancer.

There are several problems here however:

1. As we had demonstrated in some of our prior analysis, blood borne cancer cells are rare, but more importantly they are cells which are coming from and going to organs. Namely they are in transit, from whence and to where we do not know.

2. The genetic states of each of these wandering cells may be a marker of from whence it came. The problem is that we do not fully understand this genetic mutation process, and in fact as we have shown before it may actually be a Markov like chain process.

3. Understanding this change in cells may be of significant therapeutic value. However this again is uncertain given our current state of knowledge.

4. Again we come back to the cancer stem cell and ask if the few cells we find in the blood stream are the right cells to examine.

However this advance could provide significant data to allow us to expand the understanding of mutating cancer cells.

It seems that there is a significant amount of new work being done on evaluating cancers via circulating tumor cells and their DNA. Another paper in Nature states:

Cancers acquire resistance to systemic treatment as a result of clonal evolution and selection. Repeat biopsies to study genomic evolution as a result of therapy are difficult, invasive and may be confounded by intra-tumour heterogeneity Recent studies have shown that genomic alterations in solid cancers can be characterized by massively parallel sequencing of circulating cell-free tumour DNA released from cancer cells into plasma, representing a non-invasive liquid biopsy.

Here we report sequencing of cancer exomes in serial plasma samples to track genomic evolution of metastatic cancers in response to therapy. Six patients with advanced breast, ovarian and lung cancers were followed over 1–2 years. For each case, exome sequencing was performed on 2–5 plasma samples (19 in total) spanning multiple courses of treatment, at selected time points when the allele fraction of tumour mutations in plasma was high, allowing improved sensitivity.

For two cases, synchronous biopsies were also analysed, confirming genome-wide representation of the tumour genome in plasma. Quantification of allele fractions in plasma identified increased representation of mutant alleles in association with emergence of therapy resistance. ...treatment with gefitinib.

These results establish proof of principle that exome-wide analysis of circulating tumour DNA could complement current invasive biopsy approaches to identify mutations associated with acquired drug resistance in advanced cancers. Serial analysis of cancer genomes in plasma constitutes a new paradigm for the study of clonal evolution in human cancers.

Cancer Research UK commented on the works as follows[140]:

Scientists ... used traces of tumour DNA, known as circulating tumour DNA (ctDNA) found in cancer patients' blood to follow the progress of the disease as it changed over time and developed resistance to chemotherapy treatments.

They followed six patients with advanced breast, ovarian and lung cancers and took blood samples, which contained small amounts of tumour ctDNA, over one to two years.

By looking for changes in the tumour ctDNA before and after each course of treatment, they were able to identify which changes in the tumour's DNA were linked to drug resistance following each treatment session.

Using this new method they were able to identify several changes linked to drug-resistance in response to chemotherapy drugs such as paclitaxel (taxol) which is used to treat ovarian, breast and lung cancers, tamoxifen which is used to treat oestrogen-positive breast cancers and transtuzumab (Herceptin) which is used to treat HER2 positive breast cancers.

And they hope this will help shed new light on how cancer tumours develop resistance to some of our most effective chemotherapy drugs as well as providing an alternative to current methods of collecting tumour DNA – by taking a sample direct from the tumour – a much more difficult and invasive procedure.

As we noted in a previous note regarding the same set of procedures by others researchers this is a useful method to detect the progression of cancer.

However the following observations are of note:

1. Are these coming or going cells, namely are the cells on their way to a metastasis or the result of one.

2. Can we use these cells to determine the changes in DNA expression as the cells progress.

3. How effective a prognostic tool are these measurements.

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[140] http://www.cancerresearchuk.org/cancer-info/news/archive/pressrelease/2013-04-07-simple-blood-test-to-track-tumour-evolution?rss=true

4. What therapeutic methods can be applied now knowing this information.

Thus is this data of primary use or secondary. Notwithstanding its clinical use it does represent an excellent tool for genomic progression.

10.14.3Non Invasive Methodologies

Molecular Functional Imaging, MFI, provides a set of non-invasive methodologies to ascertain pathway dynamics as well as changes in genetic expression[141]. As Glunde et al state:

Molecular–functional imaging (MFI) can be defined as the noninvasive visualization of molecular and functional pathways in the tissue of interest.

As Glunde et al state:

Molecular imaging of cancer detects single molecules or their activity within cancer cells in culture or within a solid tumor. These molecules can be overexpressed receptors, activated enzymes or relocated molecules, each of which plays important roles in signaling cascades or regulatory programs that are deregulated in cancer. These in turn give cancer its phenotypic characteristics, such as evasion of apoptosis, self-sufficiency in growth signals, insensitivity to anti-growth signals, sustained angiogenesis, unlimited replicative potential, invasion of tissue and metastasis.

They continue focusing on the ability with MFI to monitor pathway dynamics:

The ability to image gene expression, promoter activity, and transcriptional activity in vivo is important because these are the starting points for many deregulated pathways in cancer. Reporter genes that are typically used include luciferase genes for bioluminescence imaging, fluorescent-protein genes for fluorescence imaging, herpes simplex virus thymidine kinase (HSVtk) genes for PET and SPECT imaging and ferritin genes or chemical exchange saturation transfer (CEST) reporters for MRI. These reporter genes are placed under the control of a promoter of interest so that promoter activity in vivo can be evaluated.

Triple-fusion-reporter genes that allow for in vivo multi-modality imaging with bioluminescence, fluorescence and PET have recently been developed. Imaging gene expression has helped to delineate mechanistic and functional aspects of oncogenes, such as myc, and tumor suppressor genes, such as p53. Another important application of imaging gene expression is monitoring viral vector delivery in vivo for future gene therapies.

10.14.3.1 Pathway Expression Estimation

[141] Further consideration of the material in this section was provided by discussions with Dr. Annick Van den Abbeele at Dana Farber Cancer Institute, Boston, MA.

Glunde et al discuss two specific genes; myc and p53. Let us first consider what they say about myc:

The myc oncogene is one of the most commonly activated oncogenes associated with the pathogenesis of liver cancer. In vivo bioluminescence imaging of transgenic mice conditionally expressing tetracycline-inducible myc proto-oncogene in liver cells proved that myc oncogene inactivation resulted in dormancy as long as myc remained inactive but that myc reactivation immediately restored the neoplastic features of previously differentiated hepatocytes and biliary cells. In this study, myc activation probably caused malignant expansion of immature liver cells with stem-cell like properties, supporting the hypothesis that liver tumors can arise from cancer stem cells.

The cancer stem cell issue is also a significant one in this analysis. We have discussed cancer stem cells previously and they are key factors in assessing metastatic behavior. However they are also of low density and generally difficult to describe genetically.

Glunde et al then progress to a discussion of p53:

The p53 tumor-suppressor gene, which is mutated in rv50% of all human cancers, plays a key role in cell cycle regulation and apoptosis following DNA damage by functioning as a sequence-specific transcription factor. Bioluminescence imaging was employed for the noninvasive evaluation of the transcriptional activity of p53 in vivo in a transgenic mouse model that conditionally expressed the firefly luciferase gene upon activation by a p53-responsive promoter. After exposure to ionizing radiation, the in vivo p53 transcriptional activity displayed a distinct oscillatory pattern, confirming p53 transcriptional oscillations previously observed in cultured cells. In vivo bioluminescence imaging of transgenic mice will prove useful in future studies assessing p53 response in vivo after systemic administration of novel therapeutic p53 or proteasome inhibitors or of agents modulating the response to ionizing radiation.

The following Table is an adaptation from the paper summarizing some targets for MFI:

Cancer characteristics/pathways	Molecular/cellular targets in cancer	Molecular MFI applications
Oncogenesis pathways	p53	Bioluminescence, PET
	myc	Bioluminescence
Multidrug resistance	P-glycoprotein	PET, SPECT
Apoptosis	Phosphatidyl serine	PET, SPECT, MRI, Optical
	externalization	(Annexin V)
Cell surface receptors	EGFR	PET, SPECT, NIR fluorescence
	HER-2/neu	T1-MRI, T2-MRI, PET, SPECT,
		fluorescence
	PSMA	PET, SPECT, fluorescence
Proliferation/differentiation	Thymidine kinase	PET (^{18}F-FLT)
	Telomerase	PET
Angiogenesis/lymph-	VEGF	PET, SPECT, MRI, fluorescence

angiogenesis		
Hypoxia	avb3 HIF-1	Fluorescence, PET, SPECT, MRI PET, fluorescence
Metabolism	NA	NA
ECM degradation	Cathepsin D	NIR fluorescence
	Cathepsin B	NIR fluorescence
	Matrix	NIR fluorescence
	metalloprotease 2	
	Lysosomes	Fluorescence
Invasion and metastasis	Cell labeling with	Fluorescence
	fluorescent proteins	

10.14.3.2 ECM Imaging

We have discussed the impact of the extracellular environment for melanoma in previous sections and thus being able to deal with that is critical as well. Thus as Glunde et al sate:

A growing list of imaging techniques such as differential interference contrast (DIC) microscopy, confocal reflection microscopy, second harmonic generation (SHG) microscopy, Fourier transform infrared (FTIR) micro-spectroscopy and atomic-force microscopy (AFM) (reviewed in) are helping us to understand the interaction be- tween tumor cells and the ECM. For example, Nomarski DIC optics has been used for dynamically tracking cell- induced matrix remodeling. Assembly properties of type I collagen and interstitial ECM have been studied with confocal reflection microscopy, without the need for staining the specimen. The nonlinear optical process of SHG requires an environment without a center of symmetry, such as an interfacial region, to produce a signal. SHG was proposed as a new contrast mechanism for live- cell imaging and was extended to image endogenous structural proteins such as those within collagen-rich layers. FTIR micro-spectroscopy, which probes the vibration energy of chemical bonds, has been used for imaging the proteolytic activity of matrix metalloproteinases (MMPs) produced by invasive cancer cells on collagen-based matrices

10.14.3.3 Next Steps

Glunde et al list the following next steps for MFI:

- *Further development of novel strategies to detect and image specific pathways and targets is required.*
- *The low concentration of receptors and molecular targets and the inherent insensitivity of imaging techniques, such as MRI, impose limitations, which require amplification strategies for increasing the sensitivity of detection.*
- *Intracellular access of reporter molecules or the internalization of reporters is necessary for imaging several critical pathways and molecules and presents another challenge.*

- *As the acquisition of multi-parametric and multi-modality images becomes increasingly routine, integrated multi-modal approaches will present unique technical and computational challenges as well as exciting opportunities.*
- *The limited translation of these approaches to clinical use presents the major challenge to date.*

The summary above clearly indicates a significant potential if the challenges are met but they also present a significant set of current limitations.

With molecular functional imaging it is possible to do the following:

1. Target specific pathway modalities so as to ascertain the state of the cells in any part of the body and to determine the mutation profile of the malignancy. Specifically we can determine the progression from the initial mutation to subsequent ones. It is suspected that mutation profiles will be of great assistance in both prognostic as well as therapeutic approaches.

2. Spatial progression will be able to be identified, quantified and projected. True assessment of metastatic movement can be made at the molecular cell level.

3. Temporal changes will be the most critical of measurements. We will be able to examine rates of change both in metastasis as well as in response to therapeutic advances.

MFI can provide a critically impressive capability which when combined with the modeling described herein will allow for improved prognostic capabilities as well as improved therapeutics.

11 MELANOMA THERAPEUTICS

There is an explosion of new cancer therapeutics. About ten years ago we saw imatinib for CML and now we have quite a few for metastatic melanoma, once a terminal disease for certain. In the Melanoma case we see some 20% may survive for extended periods of time. However the average life extension may be only 6 months at a cost that may exceed $100K. In addition there may need be several of the specific therapeutics used at one time.

A former Administration Health Care adviser has written on this of late[142]:

Many cancer patients, after getting a diagnosis of a terrifying disease, pursue any potentially promising therapy, regardless of the price. But the main cost driver is the fee-for-service payment system. The more doctors do for patients, the more reimbursement they receive. Surgeons earn more for every procedure. Oncologists typically make more money if they use newly approved drugs and the latest radiation treatments than if they use cheaper, older alternatives that work just as well. (This is because they get paid back the cost of the drug, in addition to an extra 6 percent of that cost — the more expensive the drug, the higher the compensation.)

His point is the 6% on the $100K charge. That is $6K per patient per six months. Take melanoma. The incidence is about 75K per year. Of that some 12K to 20K it the drug profile. At say $100K per person and assuming all persons, 20K, we would have in any one year $2B in costs and $120M paid to Oncologists. Is that too much? I guess it depends if you are in the 20% or the 80%.

He suggests changes:

First, over the next few years, the payment system needs to move away from fee-for-service toward a system of bundled payments, in which doctors are paid one fee for all the treatments involved in caring for a cancer patient.

This is a point well taken. But the problem is the way we compensate people based upon past assumptions.

Second, insurers have to give physicians information about where they are spending money.

I would suggest the patient also be informed. Patients all too often assume that the costs of the medication are low. They have no idea what the costs are. Moreover the basis for the costs should also be known. One must remember that the drug companies have gone through multi-Phase trials of hundreds of patients each at tens of thousands per patients just to the management of the Trial. Recall that the CROs, the Clinical Research Organizations, generate almost $30B in annual revenue just managing the Trials to comply with the FDA. That is not money in the pockets of the Pharmas.

[142] http://opinionator.blogs.nytimes.com/2013/03/23/a-plan-to-fix-cancer-care/

Third, any change in payment methods must be accompanied by rigorous quality monitoring to ensure that there is neither under- nor over-utilization of care.

Quality, now just what do we mean by that? This is what drove the character nuts in the Zen and the Art of Motorcycle Maintenance. Really, is it nothing more than what is in the eye of the beholder?

Fourth, we need more "high touch" oncology practices. In these practices, nurses manage common symptoms before they escalate to the point that they require visits to the emergency room...

Part of this is that the Oncologists are dealing with a mass amounts of new and different genetically targeted drugs which address pathways that they may have never been exposed to in Medical School. One melanoma drug leads to a new skin cancer, an unexpected effect.

Fifth, we need better incentives for research. Many expensive tests and treatments are introduced without evidence that they improve survival or reduce side effects, and with poor information about which patients should receive them.

Here I would disagree. The Trials are somewhat extensive but when you apply something used over 600 people to 20,000 you get a whole new set of issues. A drug may have to be withdrawn.

One key question is who should receive the new therapeutics? How do we manage them? Cancer is terrifying to the patient. But we now have an environment where people can find out about these new medications and demand them. Physicians are then pressed to use them, albeit with little significant survival benefit, on average. Yet that 20% who do survive contain valuable information for the next step.

Thus do we view use of the new therapeutics as the cost of continuing research or the cost of providing care?

Let us begin by recalling the specific characteristics of what cancer is. As Hanahan and Weinberg state:

The hallmarks of cancer comprise six biological capabilities acquired during the multistep development of human tumors. The hallmarks constitute an organizing principle for rationalizing the complexities of neoplastic disease. They include

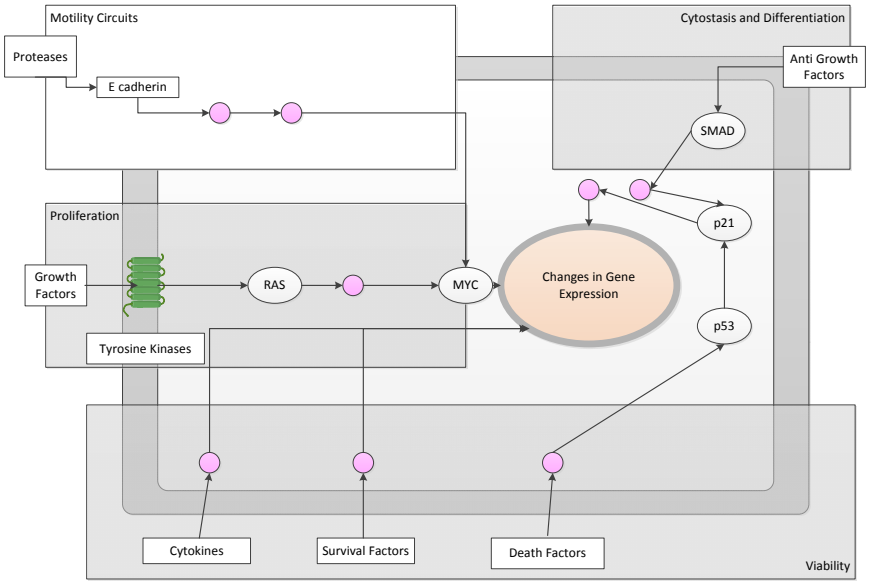
1. *sustaining proliferative signaling,*
2. *evading growth suppressors,*
3. *resisting cell death,*
4. *enabling replicative immortality,*

5. *inducing angiogenesis, and*

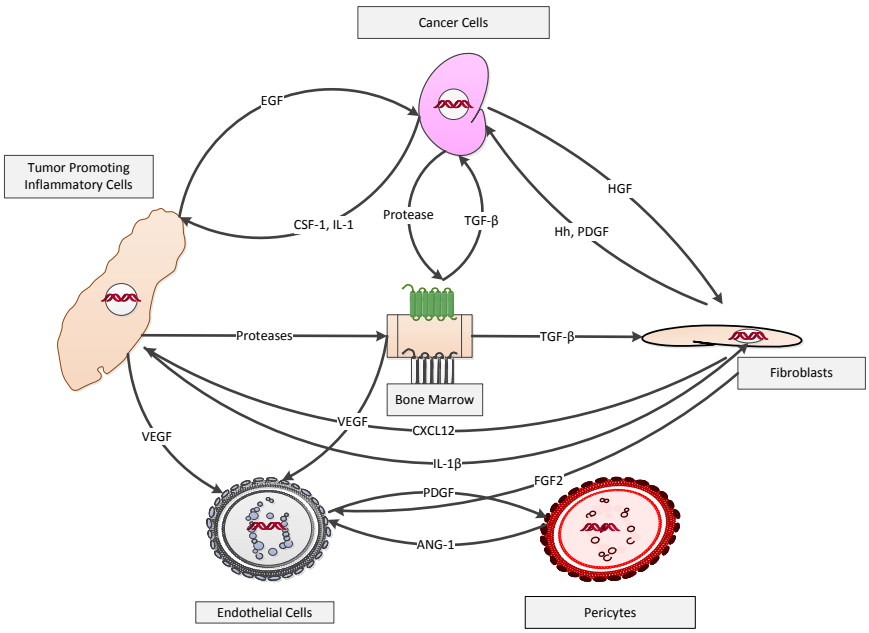
6. *Activating invasion and metastasis.*

Underlying these hallmarks is genome instability, which generates the genetic diversity that expedites their acquisition, and inflammation, which fosters multiple hallmark functions.

The Figure below is a depiction of these processes based upon the above mentioned paper. Note that this is a view of cancer from within the cell. The cell has the four characteristics shown: (i) motility, namely cancer cells move about such a melanocytes in melanoma in situ, where they move from the basal layer, (ii) proliferation, where they have activate mitotic behavior, as shown in the RAS-MEK pathway, (iii) differentiation, whereby the cell loses its functionality and becomes a nonfunctioning malignant cell, and (iv) loss of apoptosis, the cell essentially becomes immortal.



These characteristics map well onto the Hanahan-Weinberg terms. Cancer cells take on a life of their own. Therapeutics can then either attack them on the basis of the change in functionality or attack them outright. Classic chemotherapy used a meat cleaver approach, attacking any and all proliferating cells, including for example hair cells.



Thus the above depicts a somewhat global interrelationship between the cancer cell and the other cells within its environment. One of the key observations is that the cancer cells can also often take advantage of the surrounding cells and enlist them in the malignant cells own care and keep.

We are currently focusing on melanoma and from NCI we have the following estimated new cases and deaths from melanoma in the United States in 2013[143]:

- New cases: 76,690.
- Deaths: 9,480.

Furthermore the current therapeutics available, as described by NCI, are given as follows[144]:

Some melanomas that have spread to regional lymph nodes may be curable with wide local excision of the primary tumor and removal of the involved regional lymph nodes. A completed, multicenter, phase III randomized trial of patients with high-risk primary limb melanoma did not show a benefit from isolated limb perfusion with melphalan in regard to disease-free survival (DFS) or overall survival (OS) when compared to surgery alone.

Systemic treatment with high dose and pegylated interferon alpha-2b are approved for the adjuvant treatment of patients who have undergone a complete surgical resection but are considered to be at high risk for relapse. Prospective, randomized, controlled trials with both agents have shown an increase in relapse-free survival (RFS) but not OS when compared with observation.

143 http://www.cancer.gov/cancertopics/pdq/treatment/melanoma/HealthProfessional

144 http://www.cancer.gov/cancertopics/pdq/treatment/melanoma/HealthProfessional/page4

Clinicians should be aware that high-dose and pegylated interferon regimens have substantial side effects, and patients should be monitored closely. Adjuvant therapy with lower doses of interferon have not been consistently shown to have an impact on either RFS or OS.

Although melanoma that has spread to distant sites is rarely curable, both ipilimumab and vemurafenib have demonstrated an improvement in progression-free survival (PFS) and OS in international, multicenter, randomized trials in patients with unresectable or advanced disease, resulting in U.S. Food and Drug Administration (FDA) approval in 2011.

Vemurafenib is a selective BRAF V600E kinase inhibitor, and its indication is limited to patients with a demonstrated BRAF V600E mutation by an FDA-approved test.

Interleukin-2 (IL-2) was approved by the FDA in 1998 on the basis of durable complete response (CR) rates in a minority of patients (0% –8%) with previously treated metastatic melanoma in eight phase I and II studies. No improvement in OS has been demonstrated in randomized trials.

Dacarbazine (DTIC) was approved in 1970 based on overall response rates. Phase III trials indicate an overall response rate of 10% to 20%, with rare CRs observed. An impact on OS has not been demonstrated in randomized trials.

Temozolomide, an oral alkylating agent, appeared to be similar to DTIC (intravenous administration) in a randomized phase III trial with a primary endpoint of OS; however, the trial was designed for superiority, and the sample size was inadequate to prove equivalency.

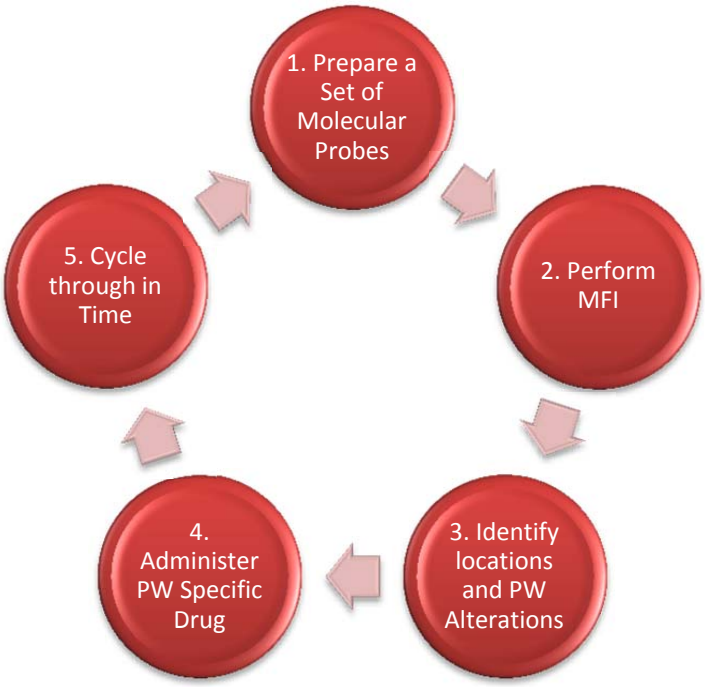
Thus there are now a significant number of options for treating melanoma, classic ones using alkylating agents and interferon or Interleukin-2, and more recent one based upon an understanding of pathways and the details of the immune system.

The classic therapeutic paradigm was to treat the disease systemically, namely suppress proliferation of cells, everywhere. Thus with something like methotrexate the cells stopped everywhere, including hair, and thus the patient went through an exhaustive and debilitating process. It also often times resulted in at best a suppression of the tumor for a short while and then a recurrence and death. With melanoma metastasis the process was often futile. Even with the early immune system approaches using interleukin and interferon, the side effects were present and the approach was for the most part systemic.

In the past decade with the understanding of pathways, in understanding the details of the immune system, and in being able to design targeted approaches to treatment we have now therapeutics that target the melanoma cells and not the entire system, almost.

The introduction of the use such as imatinib, a kinase inhibitor, for CML was a door opening step to dealing with cancers as genetically altered cells. Imatinib works to a degree, and when it fails another pathway element must be deployed.

We argue herein that there will be a new paradigm, which we depict below. It will be a paradigm based upon an understanding of cellular dynamics targeted at specific cells.



Namely:

1. Prepare a set of molecular probes which can tag the breakdown of specific pathway elements know to be specific to the metastasized cells. BRAF is but one example, and PTEN, cMyc, p53, are just a few others.

2. Perform a molecular functional imaging of the patient. This then allows for an identification of the location of the lesions, an assessment of the metabolic activity, and a clear indication of the gene expression aberrations in the tumor load.

3. Identify the specific localized PW aberrations and locations.

4. Prepare and administer therapeutics designed specifically to counter these aberrations.

5. Monitor patient and reiterate on periodic basis.

This is but one of the approaches, but it is an approach based on detailed understanding of the underlying malignancy at the gene level.

11.1 Approaches for Therapeutics

The key question is; how does one develop a therapeutic for melanoma, as an example? The answer as with many such questions is; it all depends. There are several approaches as suggested by the Hanahan and Weinberg updates. Let us summarize a few:

1. Pathway Modulators: The assumption in this class of therapeutics is that we understand the cancer at a pathway level and that there is some specific point or collection of points in the pathways which are malfunctioning. We assume we can identify that malfunction and then we further assume we can develop a therapeutic to modify the malfunction to align with the proper homeostasis of the cell.

2. Immunological Control: This approach uses the immune system but does so with specific emphasis on the uniqueness of the tumor cells. If we can identify specific cell surface markers that more accurate targeting by T cells may be achieved.

3. Oncolytic Viruses: This is a novel approach that again uses knowledge of specific cell surface markers. One can engineer viruses that attach only to malignant cells and then enter, multiply, and destroy the cells.

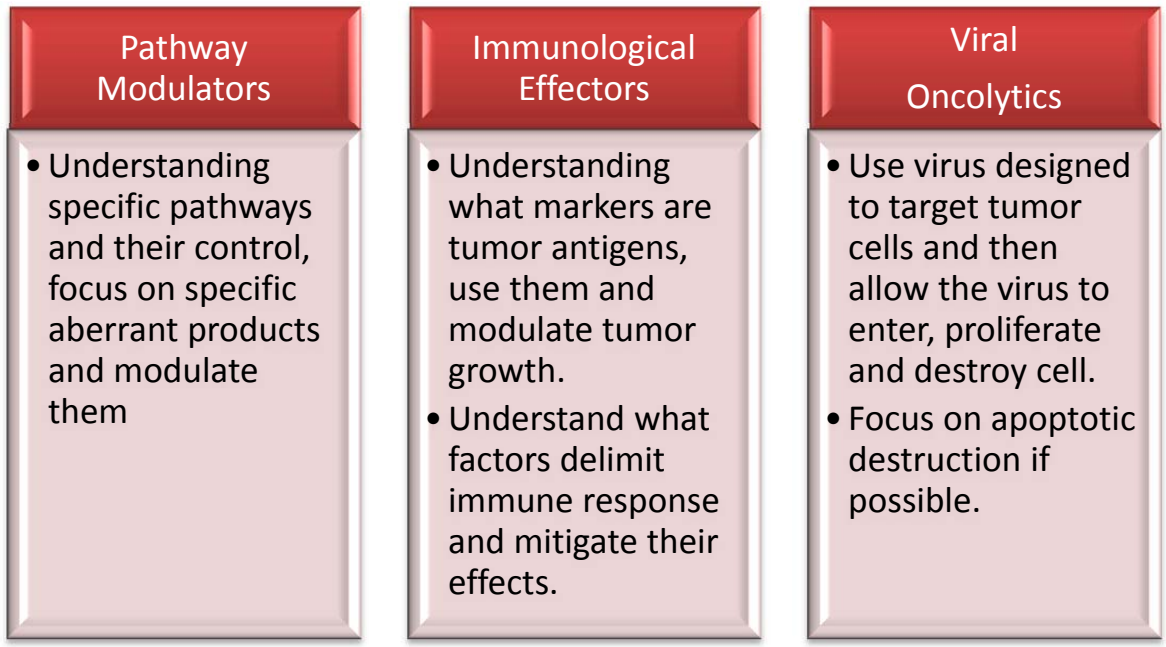
4. Extracellular Matrix Management: This is a more sophisticated approach using knowledge of the impact of the ECM on the cell.

5. Epigenetic Loss of Control: In this case we assume we are dealing with the genes in the pathways and that we have some epigenetic loss of control due to say miRNAs or methylation. Thus the therapeutic is one where we have a need to eliminate the methylation and thus reassert the gene expression or to likewise block the miRNA.

6. Gene Replacement: This approach assumes we have identified an aberrant gene, say resulting from some mutation or the like.

11.1.1 Possible Modalities

There are a set of putative therapeutic modalities for melanomas as well as cancers in general. We discuss them briefly here and detail them in the next section.

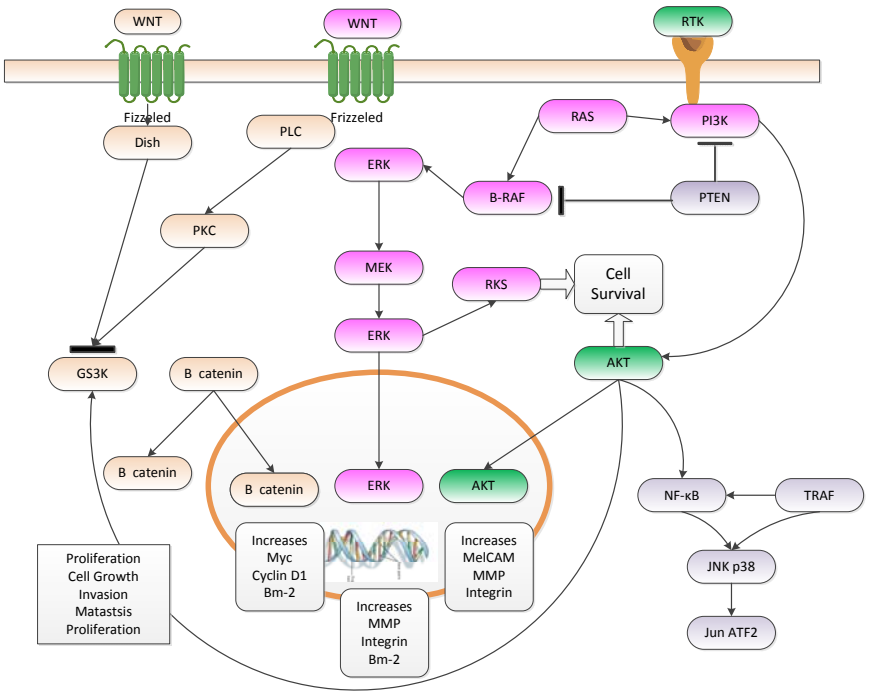


The above demonstrates the three directions we will focus on herein.

11.1.2 The Pathway Paradigm

The pathway paradigm is an articulation of our current understanding of pathways and how they can break down and result in excess proliferation, loss of functionality, movement, and all other characteristics of melanoma or cancer in general.

The following is an example of the classic pathway model as we now understand it.



Now pathways, as we have discussed in detail, control proliferation, movement, and functionality. The above is a graphic description of some of the genes related thereto.

11.2 IMMUNOLOGICAL

Using the body's own immunological system as a way to attack tumors has been an attractive option for decades. Rosenberg had been approaching this in a systematic way since the late 1960s, and as understanding of the immune system has developed there have been improved options to affect such an approach. Simply stated, tumor cells often express surface markers which are antigens which the T cells can recognize and become activated. This is why we often see clusters of lymphocytes around tumor clusters. However the tumor cells have developed means and methods to block the T cell from becoming activated and thus resulting in the digestion of the cell. The tumor cells become protected from the normal action of the immune system.

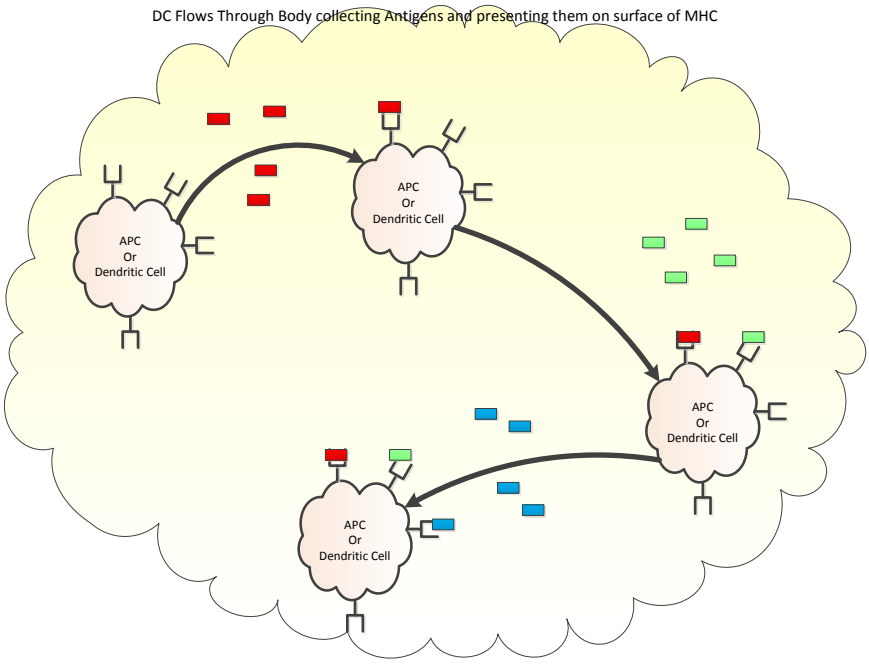
In melanoma the manner in which this happens is the use of a molecule the CTLA-4 which blocks a link normally attained with CD28 receptor. However by understanding this additional blockage one can then block the CTLA-4 from its blocking function and then allow normal operation of the immune T cell, namely destroying the melanoma cell.

11.2.1 Immunological Summary

We begin with a brief summary of how the immune system works in the case of some invasion. We assume a viral invasion but a malignant melanoma cell works the same, almost. We look at three steps.

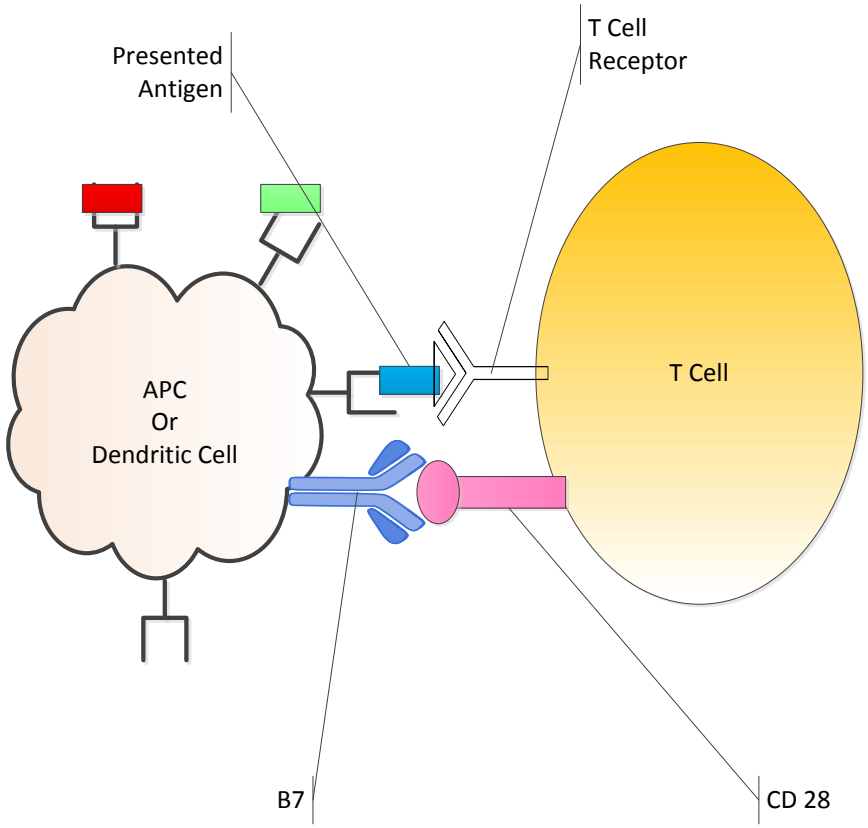
11.2.1.1 Step 1: Dendritic Cells and Antigen Presentation

First an antigen presenting cell collects antigens as it floats around the body. Seen below it collects several different types to be presented to other immune system cells. The APC or Dendritic Cells are the sensors of invaders into the body.



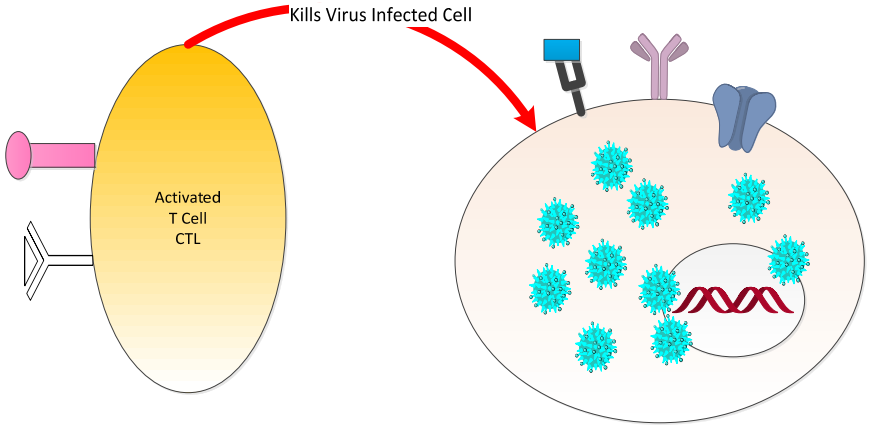
11.2.1.2 Step 2: Activation of T Cells

At a certain point the APC match up with a T cell and the antigen is then presented to the Tcell which in effect activates it to that specific "invader"



11.2.1.3 Step 3: T cell Destruction

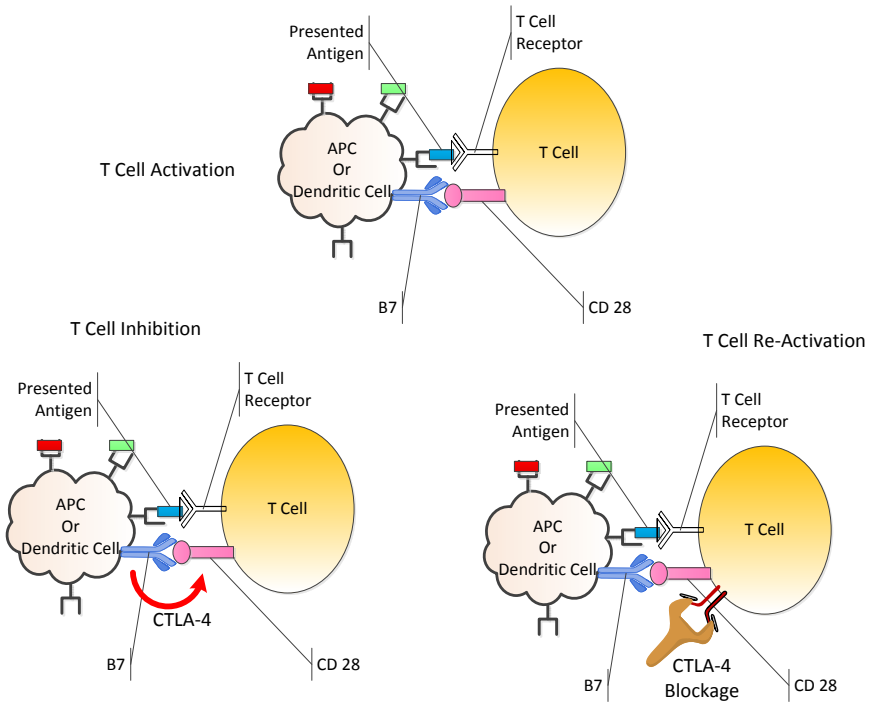
The activated T cell now can roam about activated for attaching to the invader. When such an invader, in this case a virus infected cell is seen, it attacks and destroys the cell.



11.2.2 Melanoma Paradigm

Now we will apply this basic principle to the elimination of melanoma malignant cells. The Figure below depicts the three steps that are part of this process. First the APC sees the antigen.

Now it is blocked by CTLA-4, which inhibits the destruction. Third, we find a molecule to block the attaching of the CTL-4 and thus reactivate the T cell. The 3 steps are shown below.



11.2.3 Therapeutic Action

We now use the basic principles above to describe how ipilimumab functions blocking CTLA-4.

We start by quoting Robert et al who state:

In summary, this trial showed that there was a significant improvement in overall survival among patients with previously untreated metastatic melanoma who received ipilimumab plus dacarbazine as compared with dacarbazine plus placebo. Adverse events other than those typically seen with dacarbazine or ipilimumab therapy were not identified. An increase in liver-function values is an important side effect that was observed more frequently than expected with the combination therapy.

Other ipilimumab-associated adverse events (enterocolitis and endocrinopathy) were observed, albeit at a rate that was lower than expected. The key side effects of ipilimumab were managed through adherence to treatment according to well established guidelines, including the administration of systemic glucocorticoids or other immunosuppressant agents.

Now we can examine the details of CTLA-4. As DeVita et al state:

CTLA-4 monoclonal antibody (ipilimumab) is a molecule expressed on lymphocytes that binds the B7-1 and B7-2 (CD80 and CD86) molecules on the surface of antigen-presenting cells. Engagement of the CTLA-4 molecule can suppress lymphocyte reactivity and interfere with IL-2 secretion and IL-2 receptor expression. The T-regulatory cells are the only lymphocytes in the resting circulation that constitutively expressed CTLA-4 on their surface; however, expression of CTLA-4 is transiently up-regulated after binding of the T-cell receptor. Multiple preclinical murine models have shown that CTLA-4 blockade can enhance immune-mediated tumor rejection when combined with vaccines.

Although the administration of anti–CTLA-4 monoclonal antibody to patients with metastatic melanoma has not been approved by the FDA as of the writing of this chapter, multiple clinical studies have shown that objective clinical responses can be achieved in patients treated with CTLA-4 blockade. In an updated study of 143 consecutive patients with metastatic melanoma treated with varying doses of anti–CTLA-4 either alone or in conjunction with peptide vaccination, an objective response rate of 17% was seen, including 10 patients (7%) with complete response. Substantial clinical experience with ipilimumab led to the observation that various unique patterns of clinical response could be observed in patients, including initial disease progression followed by tumor regression; mixed responses in which new lesions developed and subsequently stabilized or regressed; and late, slow continuous regression of metastatic disease.

The varied and delayed pattern of tumor response kinetics has been incorporated into strategies for clinical management of patients, for example, by observation of patients for 4-8 weeks beyond initial disease progression to detect late tumor responses. Preliminary data also indicate that a subset of patients achieving objective response or prolonged stable disease to an initial ipilimumab treatment course, who then subsequently demonstrate disease progression, can respond again to another treatment course of up to 4 doses.

*A multi-institutional prospective randomized trial was performed in 676 HLA-A*0201–positive patients with unresectable stage III or IV melanoma who received either (1) ipilimumab, (2) ipilimumab plus a gp100 peptide vaccine, or (3) the vaccine alone. Objective response rates were 11.0%, 5.7%, and 1.5%, respectively. Median overall survival was 10.1, 10.0, and 6.4 months, respectively (P = .003 for ipilimumab compared with vaccine). There were 14 (2.1%) study drug–related deaths.*

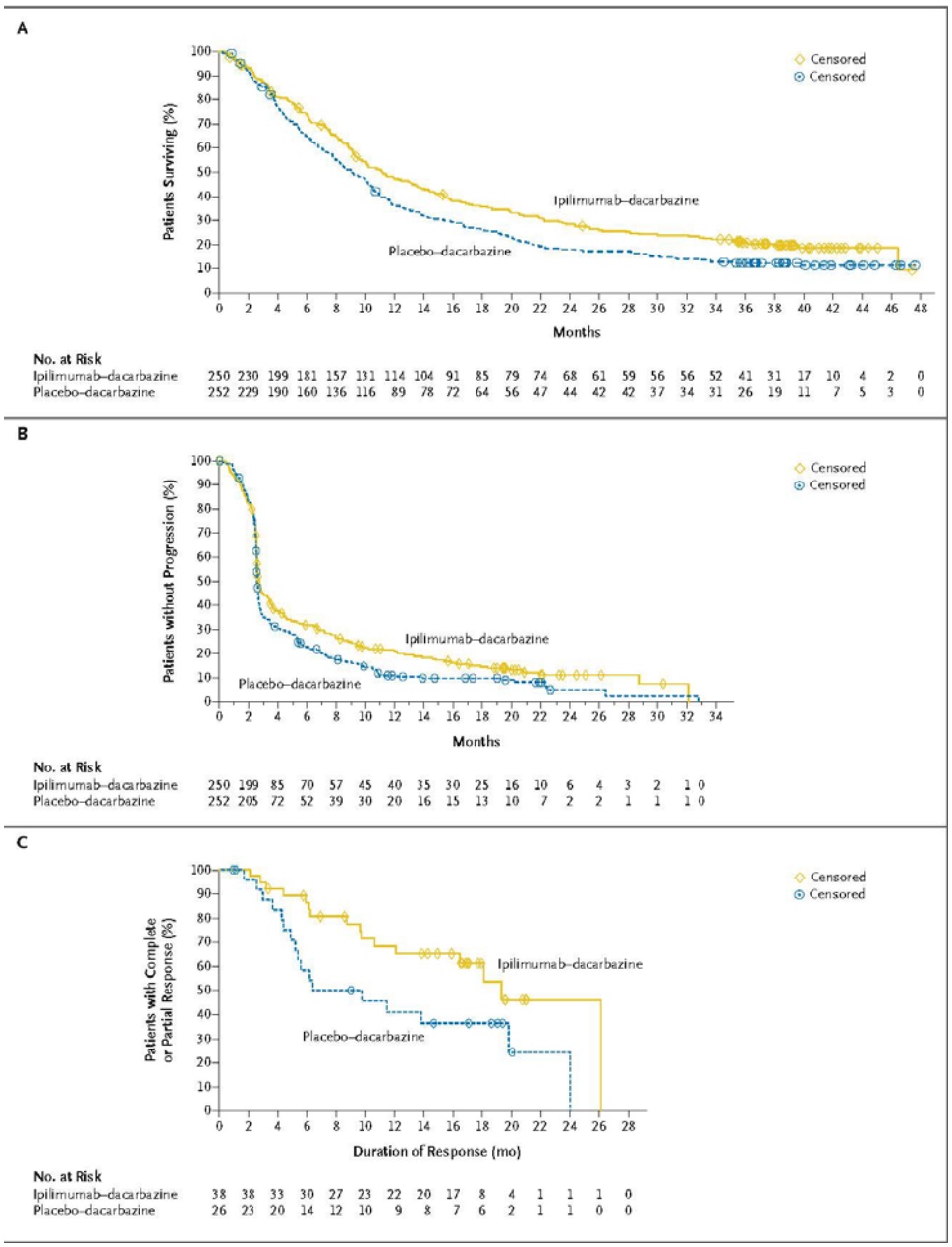
A second trial randomized 502 advanced melanoma patients without prior systemic treatment (except in the adjuvant setting) to dacarbazine (DTIC) every 3 weeks, up to 8 treatment cycles in combination with ipilimumab or placebo 10 mg/kg every 3 weeks up to 4 doses.

All patients without progressive disease or unacceptable toxicity were offered ipilimumab or placebo 10 mg/kg maintenance every 12 weeks. This trial also demonstrated improved median survival of 11.2 versus 9.1 months for patients receiving ipilimumab (P < .0009). For both randomized studies, 2- and 3- year survival estimates were approximately 10% greater for the ipilimumab containing arms compared to control.

Ipilimumab administration is associated with induction of inflammatory/autoimmune adverse events, including dermatitis; diarrhea/colitis/enteritis; and less commonly hepatitis and endocrinopathies, including hypophysitis, adrenal insufficiency and thyroiditis. Other rare autoimmune/inflammatory toxicity has been observed including nephritis, pneumonitis, uveitis, motor neuropathies, and immune-mediated thrombocytopenia. The colitis can rarely be associated with life-threatening bowel perforation.

Most of these side effects could be abrogated by the administration of steroids, although some patients may require additional immunosuppression for variable periods with anti-TNF agents. At the 3 mg/kg and 10 mg/kg dose levels of ipilimumab as a single-agent, about 15-20% and 25% of patients respectively may develop grade 3-4 autoimmune adverse events. The toxicity profile of ipilimumab may be influenced by concurrently administered agents; for example, in combination with DTIC, the expected rates of colitis/diarrhea were lower but rates of transaminase elevations were higher than expected for single-agent ipilimumab. In some phase 2 trials, a strong association was found between the probability of achieving an objective antitumor response and the development of some form of autoimmune adverse event.

The Figure below presents the Kaplan Meir curves for ipilimumab. Note that it extends survival for the 50% group to about 6 months. However there is a 20% who have indefinite survival. The question is what makes the 20% so unique and can we reproduce this.



11.3 PATHWAY MANAGED

We have examined many of the pathways which when broken can lead to tumor cells and their proliferation. We will examine several of the therapeutic possibilities here and consider future directions for development.

11.3.1 Current Developments

We begin by reviewing some of the most recent developments in pathway based therapeutics for melanoma.

As Chapman et al state:

Vemurafenib is a potent inhibitor of mutated BRAF. It has marked antitumor effects against melanoma cell lines with the BRAF V600E mutation but not against cells with wild-type BRAF. A phase 1 trial established the maximum tolerated dose to be 960 mg twice daily and showed frequent tumor responses. A phase 2 trial involving patients who had received previous treatment for melanoma with the BRAF V600E mutation showed a confirmed response rate of 53%, with a median duration of response of 6.7 months. We conducted a randomized phase 3 trials to determine whether vemurafenib would prolong the rate of overall or progression-free survival, as compared with dacarbazine.

The mechanism of the induction of cutaneous neoplasia is under investigation, but it is speculated to involve the activating effect of vemurafenib on preneoplastic cells in which wild-type BRAF is further primed by upstream pathway activation. Several investigators have shown that vemurafenib and other inhibitors of RAF kinases can potentiate the activity of the MAPK pathway in cells with wild-type BRAF.

This finding might explain the favorable therapeutic index of vemurafenib in patients who have melanoma with the BRAF V600E mutation but also suggests is further primed by upstream pathway activation. Several investigators have shown that vemurafenib and other inhibitors of RAF kinases can potentiate the activity of the MAPK pathway in cells with wild-type BRAF.

This finding might explain the favorable therapeutic index of vemurafenib in patients who have melanoma with the BRAF V600E mutation but also suggests that vemurafenib could accelerate the growth of some tumors with wild-type BRAF. An important, related ongoing effort by many research groups is to clarify how melanomas become resistant to vemurafenib. Initial studies from several groups have indicated that the MAPK pathway is reactivated in resistant tumors. Although the precise mechanisms of reactivation are still being investigated, gatekeeper mutations in BRAF, which would prevent vemurafenib from binding BRAF, have not been observed. Our results show that single-agent vemurafenib improved the rates of response and of both progression- free and overall survival, as compared with dacarbazine, in patients with metastatic melanoma with the BRAF V600E mutation. These findings provide a solid foundation for the development of future combination therapies.

As Sosman et al state:

In conclusion, this trial shows a high rate of response to vemurafenib in patients with metastatic melanoma and activating BRAF mutations. These results independently confirm the high response rate and response duration shown in a phase 1 trial. The long follow-up period in our study provides critical information on long-term overall survival, not yet shown in the phase 3 trial comparing vemurafenib with dacarbazine.19 Targeted therapy aimed at oncogenic BRAF V600 induces responses in half the patients and a median survival of 16 months.

As Flaherty et al state:

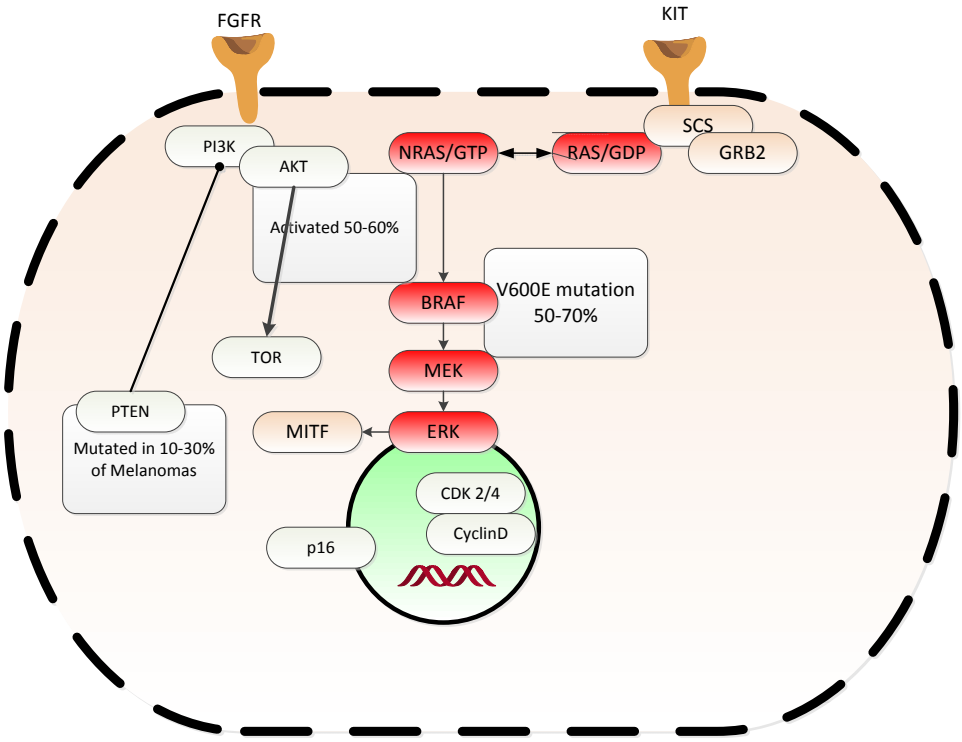
Pharmacologic inhibition of the mitogen-activated protein kinase (MAPK) pathway has proved to be a major advance in the treatment of metastatic melanoma. The use of vemurafenib and dabrafenib, agents that block MAPK signaling in patients with melanoma and the BRAF V600E mutation, has been associated with prolonged survival and progression-free survival, respectively, in randomized phase 3 trials involving patients with previously untreated melanoma. Trametinib mediates blockade of MAPK kinase (MEK), which is downstream of BRAF in the MAPK pathway and has been associated with improved progression-free and overall survival in BRAF V600 melanoma (comprising both V600E and V600K mutations).

In spite of these advances, 50% of patients who are treated with BRAF or MEK inhibitors have disease progression within 6 to 7 months after the initiation of treatment. Several mechanisms mediating resistance to BRAF inhibitors through MAPK reactivation have been described, including the up-regulation of bypass pathways mediated by cancer Osaka thyroid kinase (COT), development of de novo NRAS or MEK mutations, and dimerization or variant splicing of mutant BRAF V600. In addition, MAPK-independent signaling through receptor tyrosine kinases, such as platelet derived growth factor receptor β, insulin-like growth factor 1 receptor, and hepatocyte growth factor receptor, have been associated with resistance. New therapeutic strategies are needed to address these resistance mechanisms.

Despite successful development of oncogene targeted therapy for chronic myeloid leukemia, gastrointestinal stromal tumor, and subtypes of breast cancer and non–small-cell lung cancer, it has not yet been possible to develop combination targeted therapies that circumvent acquired resistance. The combination regimen of BRAF– MEK inhibitors described here represents a successful attempt to combine targeted therapies in an oncogene-defined patient population. Furthermore, as a consequence of unique biochemical effects observed with BRAF inhibitors, this combination appears to be associated with a reduced incidence and severity of some of the toxic effects of monotherapy with either a BRAF or MEK inhibitor. We believe that the combination of dabrafenib and trametinib warrants further evaluation as a potential treatment for metastatic melanoma with BRAF V600 mutations and other cancers with these mutations.

11.3.2 Some Pathway Issues

Let us begin by considering some specific pathways, as relates to melanoma. We show below the B-RAF pathway with a V600 mutation.



All of the pathways shown above may be affected by mutations, suppression or over activation. We discuss here basically targets of opportunity.

11.3.3 Why BRAF?

Is BRAF the most critical pathway to target or is it a target of opportunity. More than likely it is both easier to target and 40-50% of melanomas have seen this mutation. It should be noted, however, than for the Irish, it is only 10%. Now we begin by summarizing from the report by Haq et al:

Activating mutations in BRAF are the most common genetic alterations in melanoma. Inhibition of BRAF by small molecules leads to cell-cycle arrest and apoptosis. We show here that BRAF inhibition also induces an oxidative phosphorylation gene program, mitochondrial biogenesis, and the increased expression of the mitochondrial master regulator, PGC1α. We further show that a target of BRAF, the melanocyte lineage factor MITF, directly regulates the expression of PGC1α. Melanomas with activation of the BRAF/MAPK pathway have suppressed levels of MITF and PGC1α and decreased oxidative metabolism. Conversely, treatment of BRAF-mutated melanomas with BRAF inhibitors renders them addicted to oxidative phosphorylation. Our data thus identify an adaptive metabolic program that limits the efficacy of BRAF inhibitors.

As reported by Science Daily[145]:

[145] http://www.sciencedaily.com/releases/2013/03/130308103416.htm

A multi-institutional study has revealed that BRAF-positive metastatic malignant melanomas develop resistance to treatment with drugs targeting the BRAF/MEK growth pathway through a major change in metabolism. The findings, which will be published in Cancer Cell and have been released online, suggest a strategy to improve the effectiveness of currently available targeted therapies.

"We were surprised to find that melanoma cells treated with the BRAF inhibitor vemurafenib dramatically change the way they produce energy to stay alive," says David E. Fisher, MD, PhD, chief of Dermatology at Massachusetts General Hospital (MGH) and a co-corresponding author of the Cancer Cell paper. "While current BRAF inhibitor treatment is a major improvement -- shrinking tumors in most patients and extending survival for several months -- patients eventually relapse. So there is an ongoing need to improve both the magnitude and durability of these responses."

In about half the cases of malignant melanoma -- the most deadly form of skin cancer -- tumor growth is driven by mutations in the BRAF gene. Research by investigators at the MGH Cancer Center and elsewhere has shown that treatment with drugs that block BRAF activity temporarily halts tumor growth. Combining a BRAF inhibitor with a drug that targets MEK, another protein in the same growth pathway, strengthens and extends the antitumor response. The current study was designed to investigate how BRAF inhibition changes metabolic activity within melanoma cells and to find other possible treatment targets.

The most common way that cells convert glucose into energy is called oxidative phosphorylation and largely relies on the activity of the cellular structures called mitochondria. Many cancer cells use an alternative mechanism that produces the energy compound ATP without involving mitochondria. A series of experiments by the MGH team revealed that the elevated BRAF activity in BRAF-positive melanoma cells suppresses oxidative phosphorylation by reducing expression of a transcription factor called MITF.

Suppressing production of MITF reduced levels of a protein called PGC1α that regulates the generation and function of mitochondria. But melanoma cells treated with a BRAF inhibitor showed elevated MITF activity, along with increased expression of oxidative phosphorylation genes and greater numbers of mitochondria. By switching to oxidative phosphorylation to supply the energy they need, the tumor cells increased their ability to survive in spite of BRAF inhibitor treatment.

"These findings suggest that combination treatment with mitochondrial inhibitors could improve the efficacy of BRAF inhibitors in malignant melanoma," says Fisher, the Wigglesworth Professor of Dermatology at Harvard Medical School. "Several small molecules that target mitochondrial metabolism have been identified by investigators here at the MGH and elsewhere, and laboratory investigations of specific combinations of BRAF inhibitors with mitochondrial antagonists are currently underway."

11.4 Oncolytic Viral Approach

Viruses function in a manner whereby they use the host cell resources to proliferate and then spread. A virus can recognize an appropriate cell in which it can activate its reproduction via a cell surface marker and then manage its way into the cell and then capture the cell for its own purposes. A simple HPV type wart is an example. In that case we have the keratinocytes captured, and turned into a wart.

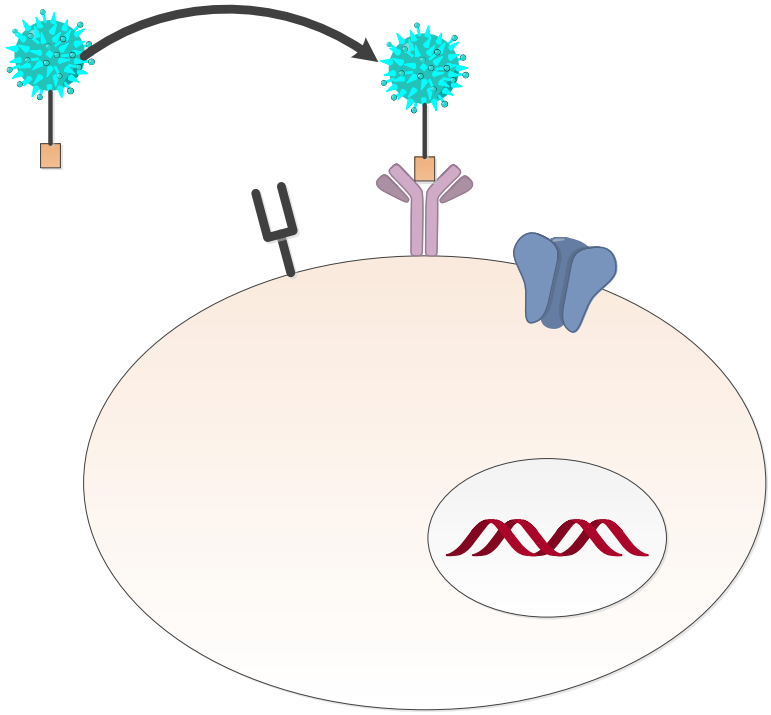
11.4.1 Oncolysis Example

We consider a simple three step process.

11.4.1.1 Step 1: Virus targets Specific Cell

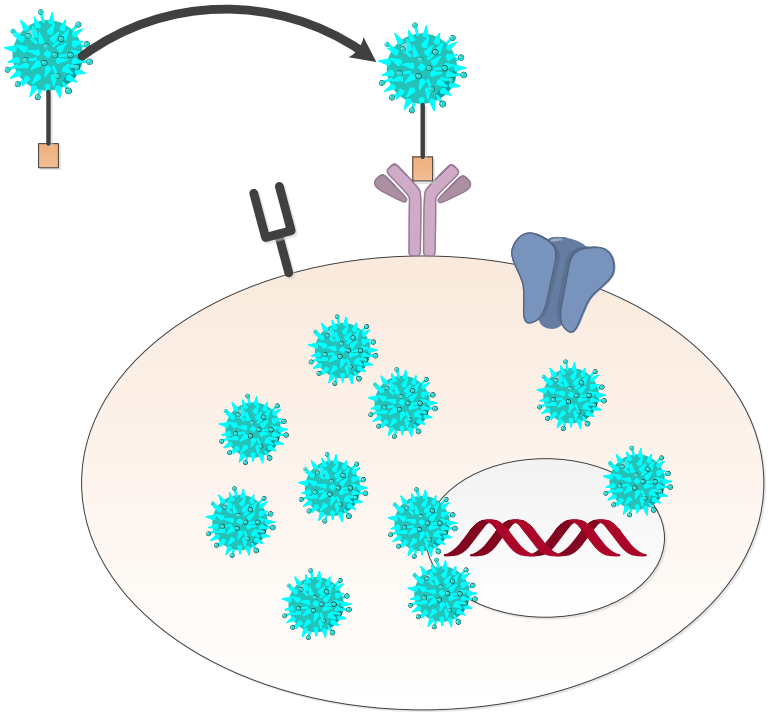
The figure below depicts an example of a virus which looks for a specific cell surface marker which it can then attach itself and enter the cell. For example HPV and HS-1 frequently attack specific epidermal cells and the generation of a wart is a classic example. The virus senses a specific cell type which it will use and then attaches and enters.

Now the problem with melanoma is that we have first to identify an appropriate cell surface marker unique to the melanoma cell and then engineer a virus to attack that specific cell. It becomes a targeted therapeutic.



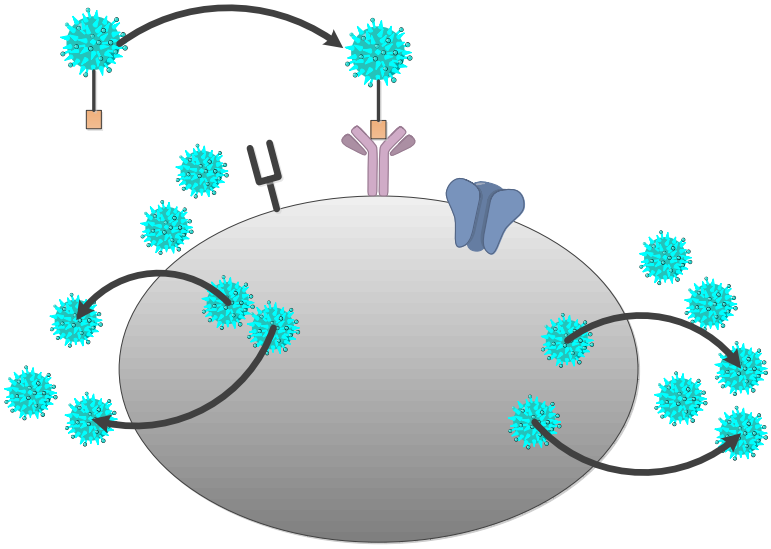
11.4.1.2 Step 2: Cell Enters and Proliferates

Viruses will then enter the cell and use the cells proteins to assist in its multiplication. In fact the cell becomes the host for this massive growth in the number of such cells.



11.4.1.3 Step 3: Virus Kills Cell

The final step is the killing of the cell by the explosive growth and expansion of the virus and then the virons go out and do the same with adjoining cells.



11.4.2 Antigens and Cell Identification

The key to identifying a tumor cell is the antigen it presents. From Abbas and Lichtman we have the following typical antigens[146]:

Type of Antigen	*Examples of Human Tumor Antigens*
Products of mutated oncogenes, tumor suppressor genes	Oncogene products: Ras mutations (~10% of human carcinomas), p210 product of Bcr/Abl rearrangements (CML)
	Tumor suppressor gene products: mutated p53 (present in ~50% of human tumors)
Unmutated but overexpressed products of oncogenes	HER2/Neu (breast and other carcinomas)
Mutated forms of cellular genes not involved in tumorigenesis	Various mutated proteins in melanomas recognized by CTLs
Products of genes that are silent in most normal tissues	Cancer/testis antigens expressed in melanomas and many carcinomas; normally expressed mainly in the testis and placenta
Normal proteins overexpressed in tumor cells	Tyrosinase, gp100, MART in melanomas (normally expressed in melanocytes)
Products of oncogenic viruses	Papillomavirus E6 and E7 proteins (cervical carcinomas)
	EBNA-1 protein of EBV (EBV-associated lymphomas, nasopharyngeal carcinoma)
Oncofetal antigens	Carcinoembryonic antigen on many tumors, also expressed in liver and other tissues during inflammation
	α-Fetoprotein
Glycolipids and glycoproteins	GM_2, GD_2 on melanomas
Differentiation antigens normally present in tissue of origin	Prostate-specific antigen in prostate carcinomas CD20 on B cell lymphomas

Now the Amgen announcement states:[147]

Amgen today announced top-line results from the Phase 3 trial in melanoma, which evaluated the efficacy and safety of talimogene laherparepvec for the treatment of unresected stage IIIB, IIIC or IV melanoma compared to treatment with subcutaneous granulocyte-macrophage colony-stimulating factor (GM-CSF).

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[146] See Abbas and Lichtman p 392.

[147] http://www.amgen.com/media/media_pr_detail.jsp?releaseID=1798143

The study met its primary endpoint of durable response rate (DRR), defined as the rate of complete or partial response lasting continuously for at least six months. A statistically significant difference was observed in DRR: 16 percent in the talimogene laherparepvec arm versus two percent in the GM-CSF arm.

The analysis of overall survival (OS), a key secondary endpoint of the study, is event driven. A pre-planned interim analysis conducted with the analysis of DRR has shown an OS trend in favor of talimogene laherparepvec as compared to GM-CSF.

"These are the first Phase 3 results of this novel approach to cancer therapy," said Sean E. Harper, M.D., executive vice president of Research and Development at Amgen. "A high unmet need exists in melanoma and we believe the innovative mechanism of action of talimogene laherparepvec may offer a promising approach for these patients."

The most frequent adverse events observed in this trial were fatigue, chills and pyrexia. The most common serious adverse events include disease progression, cellulitis and pyrexia.
Among the various types of skin cancer, melanoma is the most aggressive and also the most serious. Although melanoma accounts for less than five percent of skin cancer cases, or 132,000 cases globally each year, melanoma accounts for 75 percent of all skin cancer deaths.
Talimogene laherparepvec is an investigational oncolytic immunotherapy designed to work in two important and complementary ways - to cause local lytic destruction of tumors while also stimulating a systemic anti-tumor immune response.

We summarize this in the Table below.

Cytokine	Tumor Rejection in Animals	Clinical Trials	Toxicity
Interleukin-2	Yes	Melanoma, renal cancer, colon cancer; limited success (<15% response rate)	Vascular leak, shock, pulmonary edema
Interferon-α	No	Approved for melanoma, carcinoid tumors	Fever, fatigue
TNF	Only with local administration	Sarcoma, melanoma (isolated limb perfusion)	Septic shock syndrome
GM-CSF	No	In routine use to promote bone marrow recovery	Bone pain

11.5 OTHERS

There has been and will continue to be a growing number of therapeutics. The older ones, such as decarbazine, have been somewhat useful. Interferon, also, has been around for quite a while. Other chemotherapeutics have been tried but to no avail. The difference with the newer ones we have discussed herein is that they are based upon specific characteristics of the melanoma cell.

The trend appears to be several folds:

(i) Targeting specific pathway modifications, as they appear,

(ii) An endogenous approach utilizing the person's own immune system as a targeting vehicle,

(iii) An exogenous approach using specific cell targeted viral probes.

We assume that many of the more broadly based approaches relating to cell proliferation modulation and angiogenesis modulation will continue to be explored but with greater knowledge of the cell dynamics the approaches discussed herein will be just as powerful if not more so.

11.5.1 Classic Therapeutics

Typical classic therapeutics for the treatment of melanoma have been based upon the principles of blocking general cell proliferation. Twenty five to thirty years ago (see Fitzpatrick et al p 963, 1987) the recommended treatments were spotty at best. They used:

1. DTIC, dimethyl-triazeno-imi-diazole-carboxamide.

2. Nitrosoureas

3. Cis-platin, vinblastine, and DTIC or bleomycin

4. BCG with immunotherapy

Needless to say these had little effect, even though some survival stories were reported.

11.5.1.1 Antimetabolites

Antimetabolites generally interfere with the availability of purine or pyrimidine nucleotide precursors. They may inhibit their synthesis or compete with them in DNA or RNA synthesis. At the present time they do not seem to be effective against melanoma. Methotrexate is a classic example of the antimetabolites.

11.5.1.2 Alkylating Agents

Alkylating agents attack cells by binding to nucleophillic groups on cell constituents. They alkylate DNA and it is that process that is lethal to the cell. Alkylating agents function on proliferating and non-proliferating cells.

Decarbazine is one of the few alkylating agents used in melanoma. Several therapeutic efforts described herein use decarbazine as an adjunct. Alkylating agents become cytotoxic via a covalent bonding to nucleophillic groups on cell constituents. Decarbazine works through a metabolite not via its own properties directly.

Temozolomide is also an alkylating agent which requires a biotransformation akin to decarbazine. It also functions in a broad systemic manner and thus frequently has significant secondary effects.

11.5.1.3 Immune System Modulators

There has been an ever increasing interest in using the immune system to attack malignant cells, as intruders in the body. T cell responses and the related cytokines such as Interleukin 2 and Interferon have been an area of intense interest for well over twenty years. The early approaches as best exemplified in Rosenberg's book from 1992 describing his earliest observations and use of IL 2 and interferon.

11.5.1.4 Interleukin-2

Interleukin 2, IL-2, is a cytokine which is a driver in the proliferation, growth and differentiation of T cells. IL-2 induces the proliferation of antigen primed T cells as well as enhancing the natural killer cells, NK, for the attacking of the tumor cells.

From DeVita, Chapter 45, we have:

IL-2 was the first agent available for the treatment of metastatic cancer that functions solely through the activation of the immune system. Originally described as a growth factor for activated T cells, IL-2 was later found to exert multiple effects on cellular immune function and to induce tumor regression in mice. Subsequent clinical trials involving patients with renal cell carcinoma and malignant melanoma have demonstrated sufficient efficacy to establish IL-2 as an FDA-approved treatment for both of these malignancies.

In 1976, Morgan et al. demonstrated the existence of a growth factor present in the conditioned medium of lectin-stimulated human peripheral blood mononuclear cells that could sustain indefinitely the ex vivo proliferation of human T cells. This initial report was followed in short order by the isolation, biochemical characterization, and ultimately, the cloning of what was then termed the T-cell growth factor. Subsequently designated IL-2, this factor was shown to be a 15-kD polypeptide made up of 153 amino acids, the first 20 of which form a signal sequence that is proteolytically cleaved during secretion. Natural IL-2 is glycosylated, although the attachment of sugar moieties is not essential for biologic activity.

They continue:

IL-2 was administered at 600,000 to 720,000 IU/kg IV every 8 hours on days 1 to 5 and 15 to 19 of a treatment course. A maximum of 28 to 30 doses per course was administered; however, doses were frequently withheld because of excessive toxicity. Treatment courses were repeated at 8- to 12-week intervals in responding patients. During initial studies, patients underwent daily leukapheresis on days 8 to 12 during which large numbers of lymphocytes were obtained to be cultured in IL-2 for 3 or 4 days to generate LAK cells; these LAK cells were then reinfused into the patient during the second 5-day period of IL-2 administration.

This high-dose IL-2 regimen with or without LAK cells produced overall tumor responses in 15% to 20% of patients with metastatic melanoma or renal cell cancer in clinical trials conducted at either the NCI Surgery Branch or within the Cytokine Working Group (formerly the Extramural IL-2 and LAK Working Group).61 Complete responses were noted in 4% to 6% of patients with each disease and were frequently durable. Rare responses, usually partial and of shorter duration, were also noted in patients with either Hodgkin's or non−Hodgkin's lymphoma, or non–small cell lung, colorectal, or ovarian carcinoma.

Randomized and sequential clinical trials comparing IL-2 plus LAK cells with high-dose IL-2 alone failed to show sufficient benefit for the addition of LAK cells to justify their continued use. Because of the quality and durability of tumor responses to this high-dose IL-2 regimen, IL-2 received FDA approval for the treatment of metastatic renal cell carcinoma in 1992 and for treatment of metastatic melanoma in 1998.

Long-term follow-up data for patients with melanoma and renal cell cancer treated in the initial high-dose bolus IL-2 trials presented to the FDA have confirmed the earlier findings of response durability, with median duration for complete responses yet to be reached and few, if any, relapses observed in patients free of disease for longer than 30 months. In fact, several patients have remained free of disease in excess of 20 years since initiating treatment. These data suggest that high-dose IL-2 treatment may actually have led to the cure of some patients with these advanced malignancies previously considered incurable.

The concept of a "cure" is thus achieved in a small group of patients using this modality. From DeVita, Chapter 19, we have:

The intravenous administration of high-dose IL-2 (aldesleukin) represents an effective treatment for patients with metastatic melanoma and the treatment most likely to provide long-term complete responses and cure in these patients.

IL-2 was first described as a T-cell growth factor in 1976. The DNA sequence of the gene coding for IL-2 was determined in 1983, and soon thereafter, the IL-2 gene was expressed in Escherichia coli, produced at high concentrations, and purified to homogeneity, and the biologic characteristics of this recombinant IL-2 were determined.261 Although early studies with IL-2 used material from mammalian sources, all clinical studies of IL-2 since 1985 have used the recombinant material.

The administration of IL-2 represented the first demonstration that purely immunotherapeutic maneuvers could mediate the regression of metastatic cancer. IL-2 has no direct effect on cancer cells, and all of its antitumor activity is a function of its ability to modulate immunologic responses in the host.

The FDA-approved regimen for the treatment of patients with metastatic melanoma using IL-2 involves the use of an intravenous bolus infusion of 600,000 to 720,000 IU/kg every 8 hours to tolerance using two cycles separated by approximately 10 days (maximum of 15 doses per cycle). Results of this treatment are evaluated at 2 months after the first dose, and if tumor is

regressing or stable, a second course is then administered. This regimen was approved by the FDA for the treatment of patients with metastatic melanoma in January 1998 based on the ability of this IL-2 regimen to mediate durable responses.

The hallmark of IL-2 therapy is its ability to mediate durable complete responses in patients with widespread metastatic disease. In a report of the original 270 patients treated at 22 different institutions that was the basis of the approval of IL-2 by the FDA, a 16% objective response rate was obtained, with 17 complete responses (6%) and 26 partial responses (10%).264 At the last full analysis of these 270 patients, the median duration of response for complete responders had not been reached but exceeded 59 months, and disease progression was not observed in any patient who responded for more than 30 months.

However IL-2 has been used for several years now and does have a positive effect. Yet it is still not curative in most cases. Perhaps the characteristics of ipilimumab blocking are necessary to fine tune the approach for specific melanoma metastatic cells.

11.5.1.5 Interferon

Interferon is a cytokine and work by interacting with the surface receptors of cells. There are three types of Interferon; α, θ, γ. Interferon enhances the activity of macrophages and NK cells and increases the expression of MHC molecules and it further enhances the production of IgG2b.

As Lartigue states[148]:

Interferon clearly act upstream of many important signaling pathways, and researchers have elucidated a plethora of different cellular roles besides their namesake activity of viral interference. For example, they play vital roles in regulating both the innate and adaptive immune responses, and in the activation, migration, differentiation, and survival of various different types of immune cell. In the 1990s, the role of IFNs began to be further delineated, and there was much excitement as it became apparent that they had so-called non-antiviral effects, a variety of effects on cell growth, apoptosis, and angiogenesis (new blood vessel formation) were observed, and this is when clinicians began to realize the potential anticancer applications of IFNs.

Over the decades that followed the discovery of the cytotoxic effects of IFNs, they were touted as a potential "magic bullet" treatment for cancer. While they ultimately did not offer the cure-all that many had hoped, they did become the first treatment for numerous types of hematological cancers and solid tumors, and offered significant hope to patients. At one time or another, they were used clinically and were standard-of-care treatment for chronic myeloid leukemia (CML), hairy cell leukemia (HCL), T- and B-cell lymphomas, melanomas, renal cell carcinomas, and AIDS-associated Kaposi sarcoma.

148 http://www.onclive.com/publications/oncology-live/2013/march-2013/interferon-therapy-a-growing-family-feeds-new-interest-in-an-older-treatment/1

Thus interferons are a broad based therapeutic for many cancers and operate by exciting the immune system broadly. Yet as with any broad based therapeutic, especially one having such a strong influence on the immune system, it does have side effects. The author states:

A significant issue with type I IFN therapy is the substantial side effects experienced by patients, which include myelosuppression and nervous system disorders, and likely occur as a result of the broad cellular activity of this group of IFNs. The recently identified third type of IFNs, the IFNλs, activate similar downstream signaling pathways to the type I IFNs and have been shown to share the same biological properties, including the antitumor activity. In fact, some studies suggest that IFNλ may have even more pronounced antiapoptotic and antiproliferative effects than IFNα. Since the lambda IFNs act through a unique receptor whose expression is limited to only certain cell types, it is possible that IFNλ could offer a less toxic therapeutic alternative for certain types of cancer. This is a hypothesis that is being heavily investigated.

As DeVita et al state:

Interferon alpha-2b was evaluated in three single-agent phase 2 trials in metastatic melanoma and was associated with a 22% objective response rate among 96 patients. No randomized trial comparing interferon-α with dacarbazine in metastatic disease has been conducted. On the basis of durable responses in some patients with metastatic disease, an adjuvant therapy trial was initiated in patients with high-risk stage 2 and stage 3 melanoma. Interferon-α was administered by intravenous infusion, 20 million U/m2, for 5 consecutive days every 7 days for 4 weeks during the "induction" phase. For a subsequent 48 weeks, 10 million U/m2 were administered by subcutaneous injection on alternate days for a total of three doses every 7 days in the "maintenance" phase.

The control arm was observation, the standard at the time that the trial was conducted. Two hundred eighty-seven patients were enrolled, 80% of whom had stage III melanoma; 20% had stage IIB melanoma. Pathologic staging was performed with regional lymph node dissection because sentinel lymph node biopsy had not yet been introduced. Overall survival was the primary end point, and the trial was designed to detect a 33% improvement.

Also from DeVita et al Chapter 19 we have:

Thus, interferon has been consistently shown to improve relapse-free survival compared to either observation or ganglioside GM2/keyhole-limpet hemocyanin vaccination. The longevity of this benefit has been established with 12.6 years of median follow-up … With twice the follow-up of the initial protocol-defined analysis, the improvement in relapse-free survival continued to be statistically significant (28% reduction in risk by hazard ratio; P = .02). However, with longer follow-up or by pooled analysis of E1684 and E1690, a definitive benefit with high-dose interferon in overall survival is lacking. With long-term follow-up …, high-dose interferon was associated with a statistically insignificant 18% improvement (P = .18).

The consistency of relapse-free survival data across all trials, in the absence of a consistent or durable survival benefit, has raised speculation that interferon may contribute to causes of death that are unrelated to melanoma recurrence, such as cardiovascular disease. In addition to the

negative result for low-dose interferon in E1690, another phase 3 trial evaluated intermediate-dose interferon compared to observation in the adjuvant setting.

A total of 1,388 patients were randomly assigned to one of three arms: interferon 10 million units daily for 5 days out of 7 repeated for 4 weeks followed by 10 million units 3 times weekly for 1 year; interferon 10 million units daily for 5 days out of 7 repeated for 4 weeks followed by 5 million units 3 times weekly for 2 years; or observation. Neither interferon arm was associated with a significant improvement in the distant metastasis-free interval (7% improvement for higher dose vs. observation; 3% improvement for lower dose vs. observation). Overall survival was slightly better for the higher-dose group (5% improvement compared to observation) but not different for the lower-dose group. As a consequence, such regimens remain investigational.

More importantly we have pegylated interferon, namely using polyethylene glycol, the "peg" term, coating to protect the therapeutic from degradation before being activated within the target cell, we have from DeVita et al:

Pegylation results in substantially slower clearance of interferon after administration. This allows for more stable drug exposure than can be achieved with the shorter-lived conventional interferon-α administered on alternating days by subcutaneous injection. To achieve a similar amount of drug exposure over the course of several days, pegylated interferon can be administered less frequently and at a lower dose per injection.

This results in a lower maximum concentration after each dose while increasing the percentage of the dosing interval for which interferon is at biologically active concentrations. Per month of therapy, this regimen is less toxic than the high-dose interferon regimen tested in E1684 and E1690. However, in EORTC 18991, 1,256 patients with resected stage III melanoma were randomized between observation and treatment with pegylated interferon 6 mcg/kg once weekly for 8 weeks by subcutaneous injection followed by maintenance at 3 mcg/kg weekly for 5 years. Given the long duration of therapy, it is not surprising that the cumulative toxicities reported were only marginally less than that observed with 1 year of high-dose therapy. Nonetheless, the dose intensity achieved during the induction phase was 88% of that intended, and for the maintenance phase it was 83%.

The primary end points of the trial were distant metastasis–free survival and relapse-free -survival. Patients treated with pegylated interferon had significantly reduced risk of relapse (18% improvement by hazard ratio; P = .01), but an insignificant improvement of distant metastasis–free survival (12% improvement; P = .11). Survival follow-up was immature at the time of the analysis of the primary end points, but no significant difference in -survival was observed.

Given the substantially improved tolerability of pegylated interferon, the data supporting an improvement in relapse-free survival is being reviewed by the FDA and European regulatory authorities. Three years of pegylated (100 mcg subcutaneously once weekly) was compared to 18 months of low-dose interferon (3 million units subcutaneously 3 times weekly) in a recently reported randomized trial among 898 patients with primary melanomas greater than 1.5 mm in thickness with or without microscopic involvement of regional lymph nodes.

The peg approach is but one of several where a transport vehicle such as peg or a nano particle is used to movement of the therapeutic[149].

11.5.2 Multiple Pathways

The BRAF inhibitors can be combined with MEK inhibitors to block the progression of squamous cell cancers. However, this is a dual pathway approach but for two malignancies. The question that should be posed is; can we identify sequential pathway changes which we can then block as they occur? For example, do we expect to see changes in PTEN, p53, cMyc and other pathway elements as the tumor progresses? If so, we have certain therapeutics which may be applied to block the proliferation effects of the loss of such pathway changes.

11.5.3 Staged and Combined Therapeutics

With many cancers there has been substantial success with combined or staged use of therapeutics. Recent efforts with melanoma have tried the BRAF and immunological approaches combined with more classic approaches such as interferon. However, recent efforts to use the newer BRAF and immunological approach have met with some problems.

As Ribas et al state:

There has been great interest in testing combination therapy with the BRAF inhibitor vemurafenib and the cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4)–blocking antibody ipilimumab, currently the only two agents approved for the treatment of advanced melanoma on the basis of improved overall survival.1 Vemurafenib and ipilimumab have different mechanisms of action, and preclinical studies have suggested that BRAF inhibitors may enhance immune-cell function and antigen presentation.2-5 The only clinically significant overlapping toxic effects for these agents are in skin and liver, which rarely limit their use in patients. Therefore, ample rationale exists to investigate combined therapy with these two agents.

We conducted a phase 1 study of the concurrent administration of vemurafenib and ipilimumab. The primary goal was to evaluate safety and define an administration schedule for further clinical development. Patients were eligible to participate in the trial if they had metastatic melanoma with a BRAF V600 mutation and had not received previous therapy with a BRAF or MEK inhibitor or with CTLA-4 or programmed cell death protein 1 (PD-1)–blocking antibodies.

A second cohort of six patients was enrolled with the planned administration of a lower dose of vemurafenib (720 mg twice daily) together with the full dose of ipilimumab. Among the first four patients who were treated with this combination, elevations in aminotransferase levels (grade 3 in two patients and grade 2 in one patient) developed within 3 weeks after starting ipilimumab.

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[149] Again in the article by Lartigue it discusses such peg approaches as follows: Two pegylated IFNα agents, Pegasys and Pegintron, are approved for the treatment of chronic hepatitis B and C virus (HBV/HCV). The addition of several polyethylene glycol (PEG) molecules to IFNα helps to improve its pharmacokinetic and pharmacodynamic properties, shielding it from enzymatic degradation and increasing its half-life and stability. Since HBV and HCV infection is one of the leading causes of hepatocellular carcinoma (HCC), IFN treatment could help in the prevention of many cases of HCC, researchers hypothesize. Indeed, IFN treatment, either alone or in combination with the purine analogue ribavirin, has been shown to decrease the incidence of HCC in patients with chronic HCV and HBV.

After the toxic effects were reviewed, the remaining two patients in the second cohort received vemurafenib alone. In addition, two patients (one in each cohort) had elevations of grade 2 or 3 in the total bilirubin level with concomitant grade 3 elevations in aminotransferase levels…

Thus the simple and direct step of staging and integrating may have less than beneficial secondary effects. There may be a simple logic for each approach but the combination may introduce yet as identified responses that are not worth the use.

11.6 OBSERVATIONS

In the past few years there has been considerable success in designing and effecting therapeutics for metastatic melanoma.

We also have a set of choices. Consider the comments by Jang et al:

Patients with metastatic melanoma had few treatment options until 2011, when two drugs—ipilimumab and vemurafenib—were approved following advances in the understanding of melanoma biology and tumour immunology. Almost 50% of melanomas harbor mutations in BRAF, mainly at codon 600, which result in constitutive activation of the MAPK pathway.

The selective inhibitors of mutant BRAF Val600, vemurafenib and dabrafenib, showed major tumour responses, resulting in improved progression-free and overall survival in patients with metastatic disease, compared with chemotherapy. Antitumor activity was also recorded in brain metastases. The growth of cutaneous squamous-cell carcinomas is a unique side-effect of BRAF inhibitor therapy that is induced by the paradoxical activation of the MAPK pathway in cells with RAS mutations.

Trametinib, which targets MEK downstream of BRAF, also produced an overall survival benefit compared with chemotherapy, although tumour responses were less frequent than they were with BRAF inhibitors. Despite this robust antitumor activity, most responses to these drugs are partial and disease progression is typically seen at a median of 5—7 months. Multiple resistance mechanisms have been identified, including those that lead to reactivation of the MAPK pathway and other pathways, such as the PI3K-AKT-mTOR and VEGF pathways.

Some patients with BRAF Val600 mutant melanoma seem to also benefit from immunotherapies such as high-dose interleukin 2 and ipilimumab, which, by contrast with BRAF inhibitors, can produce durable complete responses. We review the available data to best guide initial treatment choice and the sequence of treatments for patients with BRAF Val600 mutant melanoma.

11.6.1 Extensions

This discovery leads to several observations of note:

1. One could have imagined something of this happening with Telomeres. It would almost be necessary to allow ongoing uncontrolled mitotic activity. Thus, despite the fact that there is no surprise here we do have a specific target, namely the activator of TERT.

2. Melanoma, as most other cancers, has a multiplicity of changes to genes. There are ligands, receptors, pathway elements, transcription factors, and the telomere issues as well. It is clear that no single factor is the dominant one as of yet. BRAF as a target works for a while and then there is a work around. Thus cancer is an evolving process, and one which may be highly adaptive.

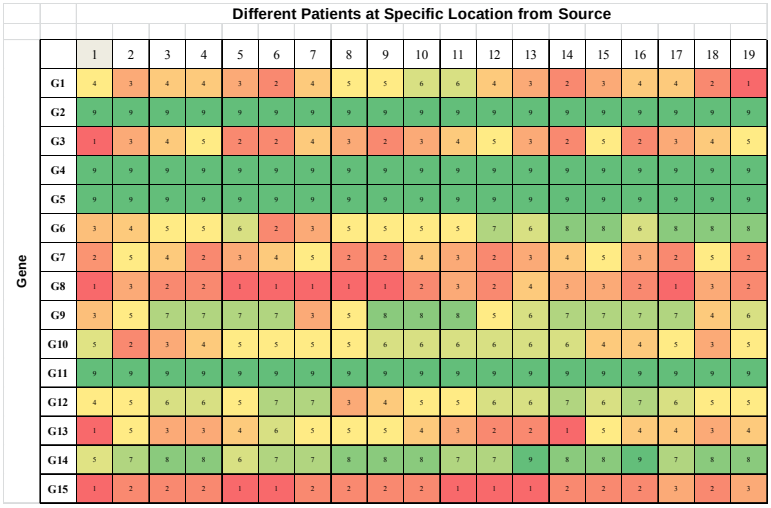
3. A Conjecture: As we have learned more and more as to aberrant genes and their products, as well as miRNAs, and their effects, one could envision several uses of malignancy profiling. We consider that in two steps.

Step 1: Profiling a Specific Patient at Various Locations. As shown below we consider a specific patient and then profile gene expression as a function of distance from the site of initiation, if such was possible. Then we can see how various aberrant genes are being expressed over the distances measure from the source. One would suspect that distance must be measured in some normalized manner but we leave that as an exercise for the student at this time. This gives us a profile for a specific patient, perhaps one for developing therapeutics.

Specific Patient at Different Locations From Source

Gene	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
G1	7	6	6	5	5	5	5	4	4	4	3	3	3	2	2	2	1	1	1
G2	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
G3	6	6	5	5	4	3	2	1	1	1	1	1	1	1	1	1	1	1	1
G4	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
G5	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
G6	6	6	6	5	5	4	4	3	3	2	2	1	1	1	1	1	1	1	1
G7	7	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
G8	7	4	4	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1
G9	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
G10	8	7	6	6	6	5	5	5	5	5	4	4	3	3	3	3	3	2	1
G11	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
G12	5	5	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
G13	6	5	4	3	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1
G14	7	7	6	6	6	6	5	5	4	4	3	3	3	2	2	2	2	2	2
G15	2	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Step 2: The Same Location but across a Large Pool of Patients: Again we look now at the same distance from the source, perhaps at the same time, again an exercise for the student, and we get profiles of the expression of aberrant genes. This allows us to understand the between patient differences.



3. Is Seventy Enough? The study did an analysis on 70 lesions. Perhaps that is not enough. Furthermore based upon our previous comments perhaps a correlative study is demanded as well, by patient and by distance.

4. One of the problems I see is the continually hyping of the results as if this is finally the right answer. Anyone even slightly familiar with the field will understand that each input is vital but assembling them in a cohesive whole is essential. The systems approach is the sine qua non, but that cannot be done without the continual bench work required to understand the details.

For example in an article in the Boston Globe the reporter states[150]:

Now scientists working independently in Boston and Germany have made a surprising discovery: a set of genetic mutations found in most melanomas, the deadliest skin cancer. The presence of these mutations in the vast majority of tumors studied suggests that the researchers may have stumbled upon a fundamental mechanism involved in a hallmark trait of cancer cells—their ability to live forever—that could one day be targeted by drugs.

Outside researchers said the work, published online Thursday in the journal Science Express, is exciting because the conclusion is the opposite of what many exhaustive studies of cancers have shown.

In reality as we have discussed, it was imperative that the Telomeres be preserved in metastasis. Millions of rapid mitotic changes in a stem cell must survive and that means keeping Telomeres and that means lots of TERT. Somehow the conclusion was logical, consistent and not at all unexpected especially given what else has been found in the past decade.

The article continues:

150 http://www.boston.com/news/science/blogs/science-in-mind/2013/01/24/boston-researchers-discover-mutations-that-underlie-melanoma-junk-dna/mNIYVavGfVsvstVj5eNfzO/blog.html

Both teams zeroed in on mutations in a part of the genome called a promoter, which acts like a volume knob on a stereo to control gene activity. The gene that the promoter controlled happened to be one that has long been of interest in cancer because it creates part of an enzyme called telomerase, which enables cancer cells to continue to divide indefinitely as one of its key jobs. Still, it wasn't easy for the researchers to convince themselves that what they found, underlying more than two-thirds of melanoma cases, was real.

One would expect this and if one looks at say the miRNA discoveries, they all add up to what controls the ultimate expression of mitotic survival.

5. Theraputics: Can we expect therapeutics from this understanding? Good question. Kinase inhibitors are now well understood, one could in theory build an inhibitor here as well. Is this the target, another target, necessary, helpful, we can only guess. Yet the above Conjecture may allow for the development of a therapeutic profiling plan for melanoma and other malignancies.

11.6.2 A System View of Therapeutics

We have developed models for cancer cell propagation and mutation. These models are based upon physical principles but clearly require experimental validation and verification. As one would expect, they are most likely first generation techniques, rough and requiring significant iterative modifications. However they are a paradigm for development.

They are also a paradigm for measuring progression and for determining what cellular modifications are where and when and thus being able to determine the best therapeutic practice.

In previous work we have developed a detailed temporal-spatial model for the propagation of cancer cells in a metastatic environment. We have also combined with that the effect of cellular mutations or equivalent expression changes, perhaps driven by epigenetic factors. We have further suggested that using molecular functional imaging that we could effectively profile the metastatic behavior consistent with the model. Having such a non-invasive data set, taken temporally over some period, could provide a powerful model for prognostic as well as putatively therapeutic usages.

Thus unlike the microarray approach, which is invasive, the molecular functional imaging, MFI, approach could provide a methodology that enable whole body assessment of the progression of the metastasis as well as the genetic alterations which are following the change.

12 CONCLUSIONS

We have examined a model for the development and spread of melanoma. The model is on the micro and macro scale and it allows for prediction based upon measured levels of selected markers. Thus we have met one of our objectives; observability. Now we have seen of late that there are drugs targeted at BRAF (V600) which is a mutated version of BRAF. It blocks the path, for a while. Combined with MEK it blocks other pathways. Thus we have a level of controllability. The model we have presented may offer global controllability, it yet requires validation.

Now there are epigenetic factors and unknown factors yet to be determined. Can we treat then as noise, unknown disturbances or totally neglect them?

12.1 SUMMARY OF PROGRESS

We have done the following in this work:

1. Examined the know processes in normal DNA action and related this to the cell cycle. This provides an up-to-date basis for understanding the stasis and proliferation of cells.

2. Examined the histological structure of melanocytes in their benign and malignant modes.

3. Developed a basic structure for pathways.

4. Analyzed all key elements related to melanoma pathways both intra and inter cellular. This included ligands, receptors, pathway elements, and transcription factors.

5. Developed a methodology to understand how to convert pathway motifs into pathway dynamics.

6. Developed and demonstrated a fully distributed model for understanding metastatic development.

This volume has not produced a detailed model that was beyond the intended scope. What we have set out to do is to frame a problem, the problem being the development for a prognostic and predictive model for melanoma. Thus one may ask if it is then possible to usefully employ genetic testing for such a set of purposes. Let us examine that question in light of what we have developed.

12.2 GENETIC TESTING FOR CDKN2A/P16

Genetic testing is a really confusing issue for many. It may present a clear and present danger for a few but for most it represents possibly but one step in a multiple step change.

As reported by Aagard on some recent research[151]:

[151] http://www.eurekalert.org/pub_releases/2010-12/uouh-pfg122110.php

The study, led by Sancy A. Leachman, M.D., Ph.D., of the University of Utah Department of Dermatology and Lisa G. Aspinwall, Ph.D., of the University of Utah Department of Psychology, both HCI investigators, surveyed 61 adults tested for the CDKN2A/p16 mutation that increases the risk of melanoma. Overall, 86.9 percent expressed support for melanoma genetic testing of minors. They cited the importance of risk awareness and the likelihood of improved prevention and screening behavior as reasons for their support. Participants were surveyed when they received their genetic test results and again two years later; their attitudes remained stable over that period.

"Developing guidelines for genetic testing of minors is complex and controversial," says Leachman. "But knowledge of their genetic status could help them make appropriate lifestyle decisions. For example, a child who tested positive might decide not to choose a summer job that demands lots of sun exposure, such as lifeguard."

This is from a Genetics in Medicine article. They report:

Genetic testing of minors is controversial, as ethical considerations depend on multiple aspects of the particular disease and familial context. For melanoma, there is a well-established and avoidable environmental influence and a documented benefit of early detection.... We surveyed 61 CDKN2A/p16 mutation-tested adults from two kindreds about their attitudes toward genetic testing of minors immediately post testing and 2 years later....

Overall, 86.9% expressed support of melanoma genetic testing of minors, with the importance of risk awareness (77.4%) ...

Concerns about inducing psychological distress or compromising children's decision autonomy were infrequently cited. Testing preferences did not vary by respondent age, gender, or melanoma history. ... Respondents strongly supported melanoma genetic testing of minors, with most citing improved health behavior as a likely outcome. We discuss options for melanoma genetic counseling and testing of minors.

CDKs are cyclin dependent kinases, and CDKN2A/p16(INK4a) is a cyclin dependent kinase inhibitor located on 9p21, and are employed in the cell cycle mechanism. We have discussed these in the previous chapters. These are a specific set of genetic predispositions for early melanoma if a few other steps happen. Like excessive exposure to backscatter.

However the issue is not so simple. No matter what parents should monitor children's sun exposure. Also there is the genetic information, what would a parent do differently than what they should already do. No matter what, sun exposure, prohibition of tanning, and the like is an imperative. That is true almost no matter what. One should remember that Bob Marley died of melanoma. Thus a priori there is no protection.

Does the knowledge by the child of the genetic marker pose a severe psychological burden? Perhaps, the old tale is that almost every first and second year Med student comes down with

Ebola or something like it, at least in their mind. There is a point when information has value and a point when it is merely a burden.

Testing has costs. Are the costs worth it? In this case most likely not. Reasonable behavior is warranted whether the genetic marker is present or not. So why test? Is it something that the parents want, another thing to speak of with other parents? This is clinically and scientifically interesting but it does not change behavior or outcomes, so why do it?

In NEJM there is an article by Hamburg and Collins regarding the use of genetic testing[152]. It raises the issue regarding the use of these tests in the future for personalized medical treatment.

The author's state:

Genetic tests are not perfect, in part because most gene mutations do not perfectly predict outcomes. Clinicians will need to understand the specificity and sensitivity of new diagnostics. The agency's goal is an efficient review process that produces diagnostic–therapeutic approaches that clinicians can rely on and allows companies that invest in establishing the validity and usefulness of tests to make specific, FDA-backed claims about benefits. Patients should be confident that diagnostic tests reliably give correct results — especially when test results are used in making major medical decisions.

The FDA has long taken a risk-based approach to the oversight of diagnostic tests, historically focusing on test kits that are broadly marketed to laboratories or the public (e.g., pregnancy tests or blood glucose tests); such kits are sold only if the FDA has determined that they accurately provide clinically significant information. But recently, many laboratories have begun performing and broadly marketing laboratory-developed tests, including complicated genetic tests. The results of these tests can be quite challenging to interpret. Because clinicians may order a genetic test only once, getting the results right the first time is crucial.

There are reports of problems with laboratory tests that have not had FDA oversight: women were erroneously told they were negative for a mutation conferring a very high risk of breast cancer; an ovarian cancer test, marketed before the completion of an NIH-funded study,[2] gave false readings that reportedly led to the unnecessary removal of women's ovaries; and flawed, mishandled data underlying a test for Down's syndrome were discovered only days before the test was to go on the market.

Through a process that includes opportunities for public input, the FDA will work to ensure the quality of key diagnostic tests, helping to protect patients and giving clinicians confidence that personalized medicine will lead to real health improvements.

In addition, the NIH will address the fact that there is no single public source of comprehensive information about the more than 2000 genetic tests that are available through clinical laboratories. On the recommendation of a federal advisory committee, the NIH — with advice from the FDA, other Department of Health and Human Services agencies, and diverse

[152] http://www.nejm.org/doi/full/10.1056/NEJMp1006304

stakeholders — is creating a voluntary genetic testing registry to address key information gaps. Readily available information *about these tests, including whether they were cleared or approved by the FDA, will help clinicians and consumers make informed decisions about using the tests to optimize health care.*

The registry will also support scientific discoveries by facilitating the sharing of data about genetic variants.

The issues regarding these tests can be characterized as follows:

1. Screening: The use to determine the predilection of an individual towards a certain disease. This is useful if and only if there is something that can be done to reduce mortality or morbidity. Just telling a person they may have a disease at some uncertain future date may be useless information and in fact harmful. Thus the mass genetic tests which seem commercially common may be resulting in harm rather than good.

2. Staging: There are millions of biopsies performed every year and the results sent to other physicians and in turn transferred in some manner to the patient. For example in a skin biopsy, if melanocytes have moved from the basal layer to the upper layers then this may be a sign for melanoma in situ. Thus what should one do? Standard practice is wide area excision but if one knew what pathways were activated in the cell one may have a better path to follow. Yet at this time such tests are not performed. The same could be said about cases of prostatic intraepithelial neoplasia, will it go to prostate cancer and if so will it be a virulent form? Knowing this via personalized medicine would save billions.

3. Treatment and Prevention: These steps are to some degree already underway.

Thus it is strange that we have the latter steps in progress and the initial high gain steps still at best in a formative stage. It would be useful for the FDA and NIH to look at a broader array of applying these new genetic personalized medical treatments.

12.2.1 The Impact of Full Gene Testing on Health Care Costs

In a recent JAMA article by Armstrong, the author attempts to show the potential for genetic analysis on a per patient basis as a means to reduce health care costs[153].

We have argued that advances in genetic tests and analysis can result in advances in three areas:

1. Determining predispositions and the attempt to mitigate the disease states.

2. Determining the specific abnormality or malignancies and assessing treatment protocols accordingly.

3. The establishment of genetically oriented treatment methodologies.

[153] http://jama.jamanetwork.com/article.aspx?articleid=1105076

However we are looking at two extreme situations:

First the understanding of basic genetic causes of disease inherited or set as a predisposition state. For example the heritability of Marfan or Huntington's. Also the predisposition for certain cancers.

Second, genetic changes in somatic cells to assess the state of a malignancy, such as breast cancer or prostate cancer.

The problem is twofold:

First we know some but hardly all genetically inheritable traits. In fact we are just starting to understand them. In a sense we are in year 1 of say a Framingham study for these issues and the time to determine what they are is lengthy.

Second, in the case of cancers, we need to understand the dynamics, and as recently shown in an earlier post from a NEJM article, the complexity of cancer genes from cell to cell is not understood.

The JAMA article states:

Several steps are needed to realize any potential beneficial effect of genomics on the cost of health care.

First, the development of effective clinical decision support is needed so that patients and clinicians use genomic test results appropriately. Such decision support already is available for several tests and should become a US Food and Drug Administration requirement for the introduction of new targeted therapy with a companion diagnostic test.

Second, information systems need to be adapted so that genomic information can be stored efficiently and accessed indefinitely.

Given its rapidly declining cost, whole-genome sequencing is likely to become the dominant model for germline genetic testing and can provide substantial efficiency assuming that test results can be stored and reliably accessed in the future.

Third, professional and patient advocacy organizations need to develop guidelines about how to manage genomic information unrelated to the clinical question of interest, in order to minimize the evaluation of clinically irrelevant genetic variation and wasted health care dollars.

Fourth, genomics can only reduce costs if the aggregate cost of testing is lower than the cost of the health care interventions that are used.

I would argue that there are many steps needed beyond these. First, most physicians lack a true understanding of these issues. For example as I have indicated a urologist may perform a prostate

biopsy and find highly disseminate HGPIN and then 9 months later perform a saturation biopsy in anticipation of a malignancy and find none. Why? Surgeons generally do not ask those questions, for them the patient has become a lucky end point. But why? What has genetically reversed what is assumed to be irreversible?

We need to establish large data bases readily accessible by professionals to be worked upon an jointly shared. Leverage of this type is essential. Closed data will result in slow progress, open and shared data is essential.

12.2.2 The Genome Scan Debate

Science has published a brief article by Koenig which is a well written article on the debate about the use of genetic analysis in assessing the susceptibility to certain diseases[154]. It states:

"*In the most provocative of four NEJM articles, David B. Goldstein, director of the Center for Human Genome Variation at Duke University in Durham, North Carolina, says the first 100 or so genome wide association studies (GWAS), which use gene chips to find associations between common gene variants and diseases, have identified important variants that appear to influence disease risk, but the impact of most of those variants is relatively low.*

He told Science that, once such association studies "have been run on the first few thousand patients for a given disease, there is only marginal return in pushing the sample sizes up further." Instead, he wants to shift more research to full sequencing of patients' genomes to find "rarer variants of larger effect" linked to disease."

The article continues:

"*Although it has become the focus of much public attention, predicting personal risk is not the point of GWAS, argues Joel N. Hirschhorn, a genomics researcher at the Broad Institute and Harvard University Medical School who wrote an NEJM commentary that offered a positive assessment of GWAS results. "The goal is not individual risk analysis but rather discovering the biological pathways underlying diseases," he says*."

As we have discussed before genetic techniques can serve in four areas; screening, staging, treatment, and prevention. The focus of this discussion is screening. I have had concerns about the overall view of this effort in that we already know that having a single aberrant gene is at best a mild predisposition. Take colon cancer for example.

Since the work of Bert Vogelstein at Hopkins we know that colon cancer, and now many other cancers, result after multiple sequences of genetic hits on certain cells. There may be a body wide genetic predisposition but it is the ensuing hits on the genes as the cells reproduce that result in the problem. It is the pathway issue which are of concern.

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[154] http://www.sciencemag.org/content/324/5926/448.full.pdf

Thus having a specific gene may give you a 5% increase in risk. The reality is what happens to that 5% risk over time, does it change the cell into a cancer precursor? Cancer is a progressive genetic disease that allows for unbridled cell growth. The progress to cancer is a result of the cell shutting down its growth control mechanism.

The second issue is that of say Type 2 Diabetes. If one discovers a gene for Type 2 Diabetes then the question is this a gene which allows for unstable weight growth that per se causes diabetes that works in some pathway manner to cycle up to diabetes. What is the overall process of type 2 Diabetes? One must look at this as a complex system not just as a single genetic hit.

Is this a problem for genetic testing? Hardly, this appears to be just another academic spat on the way to eventually seeing this holistically as a complex system.

12.2.3 Genetic Screening

In a recent British Journal of Cancer article by Pashayan et al the authors have performed a preliminary analysis of genetic screening of those for higher risk for prostate and breast cancers[155]. We herein look at the prostate cancer issue.

Simply stated the authors have assembled a database of genetic samples and for each have detailed the relative risk and the prevalence. Specifically:

1. They listed SNPs from the dbSNP ("Single Nucleotide Polymorphism database"). A SNP is a DNA sequence variation with a single nucleotide, ATGC, and may be in an exon or intron. Many of these variations occur.

2. The odds ratio, OR, is the odds of an event occurring in one group as compared to another. Thus we can say that if we have two groups, say group 1 which has the SNP alteration, and Group 0 which does not have the alteration, then the odds ratio is given by:

$$[p1/(1-p1)]/[p0/(1-p0)]$$

and if the odds ratio is greater than one then we have a greater chance of occurrence. Now consider two SNPs, and their respective individual and total odds ratio. Let p1 be SNP1 and p2 SNP2 and p0 be the lack of SNP1 and p00 the lack of SNP2. Then we have an odds ratio for both occurring, if independent, as:

$$[p1p2/(1-p1p2)]/[p0p00/(1-p0p00)]$$

This assumes independence and shows that the OR do not readily allow direct and simple calculation from each other separately. We of course can extend this principle to n SNPs. It is obvious

[155] http://www.nature.com/bjc/journal/v104/n10/full/bjc2011118a.html#bib28

3. Using the SNPs as a measure of increased or decreased risk, one can set a risk threshold and test those above and ignore those below.

The result is given by the authors as:

Compared with screening men based on age alone (aged 55–79: 10-year absolute risk 2%), personalized screening of men age 45–79 at the same risk threshold would result in 16% fewer men being eligible for screening at a cost of 3% fewer screen-detectable cases, but with added benefit of detecting additional cases in younger men at high risk. Similarly, compared with screening women based on age alone (aged 47–79: 10-year absolute risk >2.5%), personalized screening of women age 35–79 at the same risk threshold would result in 24% fewer women being eligible for screening at a cost of 14% fewer screen-detectable cases.

Personalized screening approach could improve the efficiency of screening programs. This has potential implications on informing public health policy on cancer screening

That is, by performing SNP analysis and ten establishing a threshold one can bifurcate the groups. One could also select groups in some graded multi-sector grouping as well.

The SNPs chose are shown in a modified form below. Many are on the same gene segment. There were a total of 31 SNPs as of the date of the paper where the odds ration exceeded 1.0.

dbSNP No.	Locus/gene	Risk allele frequency	Odds Ratio per allele
rs12621278	2q31/ITGA6	0.940	1.300
rs721048	2p15	0.190	1.150
rs1465618	2p21/THADA	0.230	1.080
rs2660753	3p12	0.110	1.180
rs10934853	3q21.3	0.280	1.120
rs7679673	4q24 /TET2	0.550	1.090
rs17021918	4q22/PDLIM5	0.660	1.100
rs12500426	4q22/PDLIM6	0.460	1.080
rs9364554	6q25	0.290	1.170
rs6465657	7q21	0.460	1.120
rs10486567	7p15 /JAZF1	0.770	1.120
rs2928679	8p21	0.420	1.050
rs1512268	NKX3.1	0.450	1.180
rs620861	8q24	0.610	1.280
rs10086908	8q24	0.700	1.250
rs445114	8q24	0.640	1.140
rs16902094	8q24	0.150	1.210
rs6983267	8q24	0.500	1.260
rs16901979	8q24	0.030	2.100
rs4962416	10q26 /CTBP2	0.270	1.170
rs10993994	10q11/MSMB	0.240	1.250
rs7127900	11p15	0.200	1.220
rs7931342	11q13	0.510	1.160
rs4430796	17q12 /HNF1B	0.490	1.240
rs11649743	HNF1B	0.800	1.280
rs1859962	17q24.3	0.460	1.240
rs2735839	19q13/KLK2,KLK3	0.850	1.200
rs8102476	19q13.2	0.540	1.120
rs5759167	22q13	0.530	1.160
rs5945619	Xp11	0.280	1.120

The procedure here is an interesting first step in the genetic testing of potential cancer patients. The process however will most likely require significant refinements. The process however will most likely require significant refinements.

Thus we can ask the questions as follows:

1. Which SNPs, say the set of some n of them, provides the best set to minimize mortality and minimize the number requiring testing?

2. Can there be some clustering of SNPs such that there are disjoint classes of individuals which get assigned to risk groups. Those in the highest receiving the most significant attention and those in the lowest receiving minimal?

3. Are the SNPs such that they are independent predictors or are there environmental or other exogenous factors which can effect SNPs alone?

4. What is the relationship between SNPs and the pathways known as part of PCa development?

5. Are there temporal changes in SNPs and is there some relationship between these temporal changes? Namely are there causal SNP changes?

6. What are the causes of the SNPs?

7. Knowing the SNPs and those with PCa, what can be determined regarding the dynamics of PCa development?

8. What is the relationship between SNPs and the prostate cancer stem cell? Does the CSC have different expressions?

There are many more questions that arise from this work.

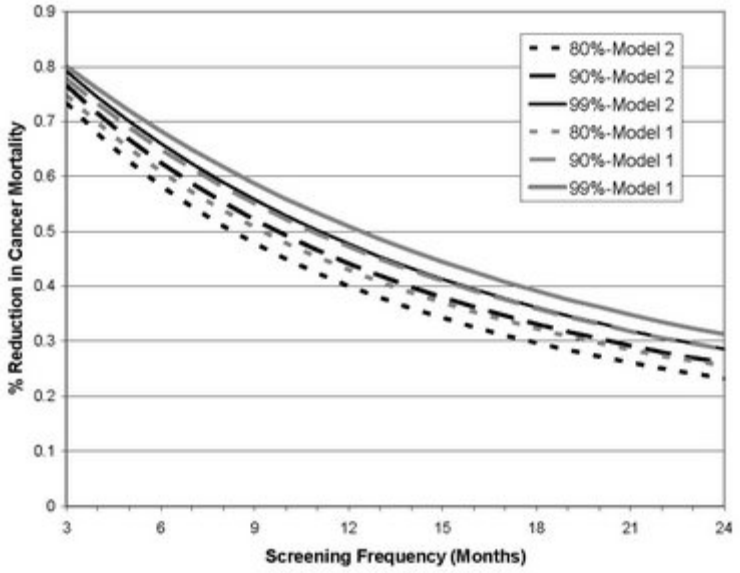
12.2.4 Cancer and Health Policy

We can now consider a slightly different issue. It is the issue of how questions, in general, are posed. In a way it is an experiment in a Fleck like world where the facts are not objectively given but collectively created[156]. As Fleck wrote his various analyses in the area of medicine, specifically with syphilis, I take this opportunity to look at cancer and screening and its implications on developing a Health Care plan.

First we present a chart on the increase in survival based on frequency of testing for ovarian cancer. This report is entitled "***Genomic Tests for Ovarian Cancer Detection and Management***" and was prepared for the *Agency for Healthcare Research and Quality* of HHS. It shows that an 80% reduction in mortality can be achieved if one screens every three months for ovarian cancer. Thus it is known what could be done. We will work through this approach again later.

[156] See Ludiwk Fleck, ***Genesis and Development of Scientific Fact***, Univ Chicago Press, 1979.

Figure 12. Effect of screening frequency on reduction in cancer mortality at different levels of test s



Let us look at another view of this same problem. Let us start with a recent article in the journal Obstetrics & Gynecology: April 2009 - Volume 113 - Issue 4 - pp 775-782. The article is entitled: Results from four rounds of ovarian cancer screening in a randomized trial. The abstract states:

"*OBJECTIVE: To test whether annual screening with transvaginal ultrasonography and CA 125 reduces ovarian cancer mortality.*

METHODS: Data from the first four annual screens, denoted T0-T3, are reported. A CA 125 value at or above 35 units/mL or an abnormality on transvaginal ultrasonography was considered a positive screen. Diagnostic follow-up of positive screens was performed at the discretion of participants' physicians. Diagnostic procedures and cancers were tracked and verified through medical records.

RESULTS: Among 34,261 screening arm women without prior oophorectomy, compliance with screening ranged from 83.1% (T0) to 77.6% (T3). Screen positivity rates declined slightly with transvaginal ultrasonography, from 4.6 at T0 to 2.9-3.4 at T1-T3; CA 125 positivity rates (range 1.4-1.8%) showed no time trend. Eighty-nine invasive ovarian or peritoneal cancers were diagnosed; 60 were screen detected. The positive predictive value (PPV) and cancer yield per 10,000 women screened on the combination of tests were similar across screening rounds (range 1.0-1.3% for PPV and 4.7-6.2 for yield); however, the biopsy (surgery) rate among screen positives decreased from 34% at T0 to 15-20% at T1-T3. The overall ratio of surgeries to screen-detected cancers was 19.5:1. Seventy-two percent of screen-detected cases were late stage (III/IV).

CONCLUSION: Through four screening rounds, the ratio of surgeries to screen-detected cancers was high, and most cases were late stage. However, the effect of screening on mortality is as yet unknown."

This is a bit obtuse for the non-professional but it displays the standard approach to the study of many disease and the efficacy of procedures used to screen for their presence and the results of actions taken thereto. The question that the researchers went out to answer was the one which says did yearly screening for ovarian cancer have any benefit. We believe in a Fleckian manner that this question and the answer could be generalized by politicians and their ilk into one which is, is screening for ovarian cancer effective. They are two different questions. We have already shown above that they are effective.

Now let us look at the data from a different perspective. Namely, in contrast to the above study let us look at the underlying "physics" of the process and look at the facts and data as say an engineer would do. Here we go with the logic:

1. We know that the incidence of ovarian cancer is 14.4% in women 45-54, 21.4% in women 55-64, 25.3% in women 65-74, and 16.3% in women 75-84. (See Berek, Gynecology, 2008). Thus there are many women who will come down with this disease, a deadly disease if caught late.

2. The five year survival for ovarian cancer is 86% at State I, 70% at stage II, 34% at stage III and 19% at stage IV. (Schorge et al Gynecology 2008 p732). Thus if one can detect the cancer at State I it is possible under current means to have 86% or better survival. Stage I means growth limited to one or both ovaries with possible growth on the surface.

3. The ovary limitation means a tumor size of 2 to 4 cm diameter at most. That is the size of an ovary and it is also the size at which one can detect the lesion on ultrasound with some specificity. Using the CA125 at a level below 35 one may get better detection but higher false alarm rates. The problem with higher false alarm rates is that it requires surgery, and although it may be performed laporascopically at first, it may or may not require full laparotomy. The latter is the case if a malignancy is detected at surgery.

4. Cancer is a disease that starts with one aberrant cell. The call multiplies and attempts to double, each division, although that is not the case in reality for a variety of known and yet to be known reasons. However, 20 doublings can occur in less than one day that is a total of 106 cells, not detectable. In 50 days we get to 40 doublings, or 1012 cells. By 125 days we get to 70 doublings, a bulky mass[157]. However for many reasons due to the individual's immune system the doubling may take longer because there may be multiple genetic steps involved.

5. Cancer masses can be detected at 108 cells by imaging and at 109 cells by palpation. At 1012 cells the patient is on the road to death from the disease[158].

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[157] Weinberg, Cancer, p 365, 2008

[158] Weinberg, Cancer, p 363, 2008

6. Thus if one performed the tests as described in the article every 120 days, then one may have a substantially improved chance of detecting the cancer at Stage I and achieving an 86% cure rate.

7. The current death rate from ovarian cancer is 8.6 per thousand females. This is a total of 280,000 women per year based upon CDC data.

8. If screening at 120 day intervals can reduce this to 42,000 deaths or equivalently save 238,000 women, at a cost of say $250 per screening or $1,000 per woman per year, over 45 years of age. The census states we have then we have a cost of 64.5 million women over 45. Thus it will cost $64.5 billion. Or, the cost per woman saved would be $271,000 per life saved per year.

First note that our simple analysis yields the same result as the HHS study we started with.

The question is it this worth it? What is a woman's life worth? Do we stop at say 65 or 75, do we continue to 85.

The other issue is that the authors of the article assumed annual testing. Based upon the logic above we see it means at least quarterly testing due to the tumor growth rate. By the way this applies to all tumors. Perhaps a study should address the question; "How frequently should testing be performed to obtain a material reduction in mortality from that disease". Clearly annual testing will at best get say one sixth of the cases; say 18% if everyone is tested.

This analysis has raised two issues:

1. When considering revising health care, what screening should be done and at what cost. Can, for example, a patient, person, pay for their own screening costs, at a price pari passu to the lowest cost paid, if they feel that they want more testing. Or will the Government as do the insurance companies today, have the lowest price forcing individual payers to subsidize the group payers, and in this case the Government. If the Government agrees to do annual testing and to be reliable it demands at least quarterly testing, then can a patient have the right to play on a level playing field or will the individual be taxed to seek better care on top of the costs?

2. When medical research is performed, there is a strong Fleck influence of a "thought collective" approach. The Fleck view of facts plays a significant role as well. The questions that should be posed are, "What level of screening result in what level of reduction in mortality?" Instead the way these are done is to take say an annual screening and determine if it is useful. The problem with this Fleckian "thought collective" approach is that it will then become part of the comparative clinical effectiveness schema as proposed by the Administration. Namely, the clinical result says that the screening is not useful. Wrong! The experiment shows that that specific type of screening is not useful.

Thus there are the above two issues of a much broader scope which can be drawn from this article, obscure as it may initially seem.

12.2.5 Melanoma and Genetic Screening

Now we can consider specific melanoma tests. As has been reported in the NY Times, there is a test for ocular melanoma, of the choroid disc. The newspaper states[159]:

"….(Mrs. X) had a new option, something that became possible only in this new genetic age. She could have a genetic test of her tumor that could reveal her prognosis with uncanny precision. The test identifies one of two gene patterns in eye melanomas. Almost everyone in Class 1 — roughly half of patients — is cured when the tumor is removed. As for those in Class 2, 80 to 90 percent will die within five years. Their cancers will re-emerge as growths in the liver. For them, there is no cure and no way to slow the disease….No test has ever been so accurate in predicting cancer outcomes, researchers said."

As Shields et al report in the original article regarding this result,

(Purpose of the analysis was…) To determine the relationship between monosomy 3 and incidence of metastasis after genetic testing of uveal melanoma using fine-needle aspiration biopsy (FNAB). … According to FNAB results, patients with uveal melanoma demonstrating complete monosomy 3 have substantially poorer prognosis at 3 years than those with partial monosomy 3 or disomy 3. Patients with partial monosomy 3 do not significantly differ in outcome from those with disomy 3.

Now the question is what value are the tests. In this case we have a test which can say whether or not you will likely survive with a high degree of accuracy. However if you fall in the surviving group, then not much more need be done and you just will make it. However if you fall in the non-surviving group nothing will help that, An excellent gene based prognostic test, yet a test where nothing more can be accomplished based upon the result other than the patient having a more reliable estimate of their ultimate destiny.

We look toward tests which allow intermediation on the side of reducing mortality and morbidity. Our analyses, our models, our approach, is only of value if it facilitates that reduction in mortality and morbidity.

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159 http://www.nytimes.com/2012/07/10/health/genetic-test-changes-game-in-cancer-prognosis.html?hp Shields, C., et al, This is from the paper by Prognosis of uveal melanoma in 500 cases using genetic testing of fine-needle aspiration biopsy specimens, Ophthalmology. 2011 Feb;118(2):396-401. See http://www.ncbi.nlm.nih.gov/pubmed/20869116

13 APPENDIX A: DEFINITIONS

1. **AKT:** A transforming serine-threonine kinase involved in cell survival.
2. **Akt pathway**: A signal transduction pathway involving the signaling molecules phosphatidylinositol-3 kinase (PI3K) and Akt, where PI3K generates phosphorylated inositides at the cell membrane which are required for the recruitment and activation of Akt, a transforming serine-threonine kinase involved in cell survival.
3. **Akt/PKB:** Protein kinase B belongs to a pathway that is responsible for cell survival. Following activation (ie, phosphorylation) by PI3K, activated Akt blocks the activity of molecules involved in the apoptotic pathway by in turn phosphorylating them.
4. **c-myc:** An oncoprotein, c-myc is a transcriptional factor that activates the transcription of growth-associated genes. Its activity is positively modulated by heterodimerization with Max.
5. **ERK (extracellular receptor kinase):** A second messenger kinase (an enzyme adding phosphate groups from ATP), ERK belongs to the MAPK family and is responsible for transmitting signals from the cellular surface to the nucleus by the activation of transcription factors, including NF-κB. It belongs to the proliferative/mitogenic signal transduction pathway activated by tyrosine kinase receptors.
6. **Erk1/Erk2 MAPK signaling pathway:** See *ERK-MAP kinase pathway*
7. **ERK1:** A member of the ERK family of protein kinases. (See *ERK [extracellular receptor kinase]*)
8. **ERK2:** A member of the ERK family of protein kinases. (See *ERK [extracellular receptor kinase]*)
9. **ERK-MAP kinase pathway:** Survival and proliferative pathways that channel signals through the activation of ERK and MAPK. (See *ERK [extracellular receptor kinase]* and *MAPK [mitogen-activated protein kinase]*)
10. **MEK (MAPK-ERK kinase):** A protein kinase, MEK is activated by c-Raf through phosphorylation of specific serine residues. Activation of ERK by activated MEK may lead to translocation of ERK to the nucleus, resulting in activation of specific transcription factors.
11. **MEK1:** An isoform of MEK. (See *MEK [MAPK-ERK kinase]*)
12. **MEK2:** An isoform of MEK. (See *MEK [MAPK-ERK kinase]*)
13. **MEK/MAP/ERK kinase:** A family of kinases that activates the MAPK family of proteins through phosphorylation of both a threonine (Thr) and tyrosine (Tyr) residue. These kinases belong to the signal transduction pathway governing proliferation. Growth factors involved in proliferative pathways such as EGF, FGF, and PDGF are the extracellular stimuli that activate these kinases.
14. **PI3K-PTEN-AKT pathway:** Signal transduction pathways involving the signaling molecules phosphatidylinositol-3 kinase (PI3K), PTEN and Akt. PI3K generates phosphorylated inositides at the cell membrane which are required for the recruitment and

activation of the serine kinase Akt. PTEN is a lipid phosphatase which counteracts the effect of PI3K. Accordingly mutated PI3K and AKT act as dominant oncogenes while PTEN is a tumor suppressor gene.

15. **PI3K:** Phosphatidylinositol-3 phosphate kinase (PI3K) adds a phosphate group to PI3, which is a downstream signaling molecule involved in survival/proliferative pathways mediated by growth factors such as the EGF and the PDGFs.
16. **PIK3CA:** The catalytic subunit of phosphatidylinositol 3-kinase involved in the generation of PIP3 which, in turn, leads to the activation of Akt and other oncogenic kinases. Mutations in the *PIK3CA* gene have been found in a number of cancers, including ovarian, breast, colon, and lung carcinomas. (See *PI3K* and *Akt/PKB*)
17. ***PIK3R3*:** Gene encoding for the regulatory unit of phosphatidylinositol 3 kinase. (See *PI3K* and *PIK3CA*)
18. **PTEN (phosphatase and tensin homolog):***PTEN* is a tumor suppressor gene with a gamut of regulatory activities. The gene product is a multifunctional molecule. The predominant activity identified for *PTEN* is its lipid phosphatase activity that converts inositol trisphosphates into inositol bisphosphates, thus inhibiting survival and proliferative pathways that are activated by inositol trisphosphates. *PTEN* acts to maintain arrest in the G1 phase of the cell cycle and enable apoptosis through an AKT-dependent mechanism.
19. **Raf:** Raf proteins (Raf-1, A-Raf, B-Raf) are intermediate to Ras and MAPK in the cellular proliferative pathway. Raf proteins are typically activated by Ras via phosphorylation, and activated Raf proteins in turn activate MAPK via phosphorylation. However, Raf proteins may also be independently activated by other kinases.
20. **Raf kinase:** Receptor activation factor (RAF) kinase, or MAPKK kinase, or MAPKKK is an essential component of the MAP Kinase pathway which is a key signaling mechanism that regulates many cellular functions such as cell growth, transformation, and apoptosis. Raf can be mutated or overexpressed in certain types of cancer. Raf kinase is a target of inhibition by sorafenib. The regulation of Raf is complex and involves the integration of other signaling pathways as well as intramolecular interactions, phosphorylation, dephosphorylation, and protein-protein interactions.
21. **Raf-1/MEK/ERK:** Signaling pathways involving Raf, MEK, and ERK.
22. **Raf-MEK-ERK pathway:** A phospho-relay system in which the three protein kinases get activated sequentially. Activated Raf activates MEK, which activates ERK.
23. **Ras:** The Ras gene family consists of H-Ras, N-Ras, and K-Ras. The Ras proteins are typically small triphosphate-binding proteins, and are the common upstream molecule of several signaling pathways that play a key role in signal transduction, which results in cellular proliferation and transformation.
24. **Ras pathway:** A signal transduction pathway involving the signaling molecules Ras, Raf, and ERK, where activated Ras activates Raf, which then activates MEK (MAPK/ERK kinase) and thereby ERK. Generally, the involvement of these molecules results in enhanced cell survival and/or proliferation.

25. **Ras-MAP kinase cascade:** Signal transduction pathway involving Ras and MAP kinase. The pathway is generally involved in proliferative and survival signals. (See *Ras* and *MAPK [mitogen-activated protein kinase*)
26. **Ras/Raf/ERK pathway:** Signal transduction pathways involving the signaling molecules Ras, Raf, and ERK, where activated Ras activates Raf, which then activates MEK (MAPK/ERK kinase) and thereby ERK. Generally, the involvement of these molecules results in enhanced cell survival and/or proliferation. Activating mutations of Raf have been discovered in some human tumors such as melanoma and non-small-cell lung cancer. (See *Ras*, *Raf*, *MAPK [mitogen-activated protein kinase]*, and *ERK [extracellular receptor kinase]*)
27. **Ras/Raf/MEK/ERK:** See *Ras*, *Raf*, *MEK (MAPK-ERK kinase)*, *MAPK (mitogen-activated protein kinase)*, and *ERK (extracellular receptor kinase)*.
28. **RASSF1A (Ras association domain family member 1A):** One of the most commonly epigenetically silenced tumor suppressor genes in human cancer that controls cell cycle and apoptosis.

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