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PROSTATE CANCER GENOMICS: A SYSTEMS APPROACH

TERRENCE P MCGARTY

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Dedication

THIS BOOK IS DEDICATED TO THREE PEOPLE:

FIRST, TO MY FATHER **TERRENCE P MCGARTY SR.** WHO DIED OF THIS DISEASE, AND WHO TAUGHT ME THAT PERSISTENCE IS THE ESSENCE OF SUCCESS;

SECOND, MY SISTER **KATHLEEN**, A SUPERB AND DEDICATED NURSE WHO DEALT WITH THOSE IN NEED, FAMILY AND PATIENTS, WELL BEYOND THE CALL,

THIRD, TO MY FIRST HEAD NURSE, **MRS. KAHN**, WHO TAUGHT ME RESPECT FOR PATIENTS AND PEOPLE, THAT EVERYONE BEING TAUGHT SHOULD HAVE SUCH.

Preface

This book has been a long and evolving process. It is also but a snapshot in time since the insight and understanding of cancer seems to be changing by the hour. One may initially ask why prostate cancer, and the answer can be embellished with many reasons but frankly it just seemed to fall into my lap as the greatest of the challenges. The work of Vogelstein two decades ago was the start of the thinking. He presented the first set of pathway dependent models. The work on CML is also a benchmark and the recent work on melanoma as well. But prostate cancer is most interesting both as a model but equally well as a cancer mixed in the political debates on health care.

As basically an engineer, I really think that way, not a scientist nor a physician, I look at systems not specific entities, all too often the people get in the way, I see that prostate cancer has been viewed by many as a single entity and massive Government bureaucratic decisions are being made that will impact the lives of many. Thus what we approach herein is an approach to a holistic problem, not just a recapitulation of what is known but a recasting of that in a systems approach, where the system is not only the patient but the patient in the society in which they find themselves.

This is not a medical work, it is not a scientific work, rather it is an engineering work. That means that certain levels of detail are irrelevant. It is akin to neglecting all the quantum effects of holes and electrons when considering a transistor circuit. Just focus on the physical and phenomenological elements at the uppermost level. The level where the details and models give reliable analytical and synthetic capabilities. One does not necessarily have to understand protein folding nor ADP and ATP interactions in any detail. Just remember that they are there and look to what is necessary to explain the phenomenon. Also look at it in a Popperian sense, namely what can make the result be proved wrong.

The work is an engineering work, namely one which tries to use the basic science and yet distill what is essential, then create models which are predictive, tests the models for deficiencies and then makes modifications. The intent from an engineering perspective is twofold; observable, namely to use measurements to the extent that we can deliver models which reflect reality and second controllable, from the models ascertain what can be used to control the result. The depth of the models must be adequate to meet the two goals.

Thus we are challenged with such effects as methylation and miRNAs, which can be handled as elements in the model or noise factors which are just so random that they are too difficult to model. In the previous statement we reflect the engineers view of probability, namely something is random if we cannot truly model it in detail but that we know it has a significant and measurable effect, and yet we know also how great a perturbation the effect may have. Thus we call it noise.

We have written previously on the genetics of plants and plant color, looking at secondary pathways and their observables, namely flower color. We have found that as one would expect

there is a great deal of complementarity between the two. We have also written on a similar vein concerning melanoma. The two cancers are quite far apart. Prostate cancer is glandular, namely an adenocarcinoma, whereas melanoma is epithelial, starting in the basal layer of the epidermis. Frequently prostate cancer is indolent but always melanoma is devastating. Melanoma can be visualized if and only if one looks, and prostate cancer can at best be inferred. They both are challenges and both show commonality.

This work has tried to remain current. However the challenge is continually one of keeping up with progress in the field, a broad field. On the research side there is often a focus on compiling new facts about known or recently uncovered genes and their products. However many of these discoveries lack a system structure, a set of temporal and spatial relationships, one that can be articulated and tested. Thus as a new set of genes or gene markers are found in some state of PCa there is often the tendency to announce them as the next best marker. PSA has often been the grail or the whipping boy of those with other agenda. PSA we believe serves a purpose, especially if one tries to understand it in a system manner.

However it is one of many such markers. We have argued elsewhere, in our book on Health Care Economics, that one of the major forthcoming advances will be the use of genomics in diagnosis and staging, and soon thereafter in detection and prevention. Yet the time is not yet here and we believe that it is essential that a systems model be available before such can be effectively accomplished.

This is a second Draft of this work. It has been considerably restructured, revised, and updated to reflect an additional two years of effort.

Terrence P McGarty
Florham Park, NJ
January 2013

Table of Contents

| | | |
|-------|---|----|
| 1 | Introduction..... | 16 |
| 1.1 | Section One: PSA and Prostate Cancer..... | 16 |
| 1.2 | Prostate Cancer and Genetic Pathways | 17 |
| 1.3 | Prostate Cancer via a Systems Approach..... | 17 |
| 1.4 | Deficiencies | 17 |
| 1.5 | The Prostate Cancer Debate | 18 |
| 2 | PSA and Its Implications | 19 |
| 2.1 | PSA Function | 20 |
| 2.1.1 | PSA | 21 |
| 2.1.2 | PSA Velocity | 22 |
| 2.1.3 | Percent Free PSA | 23 |
| 2.2 | The PSA Controversy..... | 24 |
| 2.3 | The PSA Debate | 29 |
| 2.3.1 | The American and European Studies..... | 29 |
| 2.3.2 | Summary | 35 |
| 2.4 | The Conflict in PSA Studies | 36 |
| 2.5 | PSA and Comparative Effectiveness Research..... | 37 |
| 3 | Basic Prostate Cancer Tests | 42 |
| 3.1 | Prostate Biopsy Sampling | 42 |
| 3.1.1 | Some Preliminary Facts | 42 |
| 3.1.2 | Basic Assumptions..... | 47 |
| 3.1.3 | Analysis of the Detection..... | 49 |
| 3.2 | Verification Bias | 53 |
| 3.2.1 | The Problem..... | 54 |
| 3.2.2 | Approaches to Eliminating Verification Bias | 56 |
| 3.2.3 | Bayesian Approach | 61 |
| 3.2.4 | Summary | 62 |
| 3.3 | PSA Dynamics | 63 |
| 3.3.1 | The Problem..... | 63 |
| 3.3.2 | Alternatives | 65 |
| 3.3.3 | The System Approach..... | 65 |
| 3.3.4 | Hypothesis Detection Model..... | 68 |
| 3.3.5 | Adequacy of Data in Model..... | 69 |

| | | |
|-------|---|-----|
| 3.4 | Logistic Analyses | 70 |
| 3.5 | Classification Methodologies | 71 |
| 3.6 | A Maximum Likelihood Systems Classifier | 71 |
| 3.7 | Example..... | 76 |
| 3.8 | Conclusions | 79 |
| 4 | Prostate Histology and Pathology | 80 |
| 4.1 | The Normal Prostate..... | 80 |
| 4.2 | Summary of Prostate States..... | 82 |
| 4.3 | Prostatic Intraepithelial Neoplasia | 84 |
| 4.3.1 | HGPIN Characterization..... | 85 |
| 4.3.2 | PIN Morphology | 87 |
| 4.3.3 | Some HGPIN Models | 89 |
| 4.3.4 | HGPIN, A Precursor of PCa? | 96 |
| 4.4 | PCa Histology and Grading..... | 98 |
| 4.4.1 | Grading | 99 |
| 4.4.2 | Gleason Summary | 103 |
| 4.4.3 | Models From Grading..... | 104 |
| 4.5 | Regression | 105 |
| 4.5.1 | NSAID Regression..... | 105 |
| 4.5.2 | Androgen Deprivation Therapy Regression | 105 |
| 4.5.3 | mTOR Inhibition..... | 106 |
| 4.6 | Summary | 106 |
| 5 | The Cell Cycle: A Beginning for Pathways..... | 107 |
| 5.1 | Genetic Principles and Applications | 109 |
| 5.1.1 | Chromosomes and Genes..... | 109 |
| 5.1.4 | Chromosome | 110 |
| 5.1.5 | DNA..... | 111 |
| 5.1.6 | Gene | 116 |
| 5.2 | Genotype and Phenotype..... | 117 |
| 5.3 | Genetics | 121 |
| 5.3.1 | Cells and DNA | 121 |
| 5.3.2 | Gene Processes..... | 122 |
| 5.3.3 | Activators | 125 |
| 5.3.4 | Repressors | 128 |

| | | |
|-------|--|-----|
| 5.4 | Expression Analysis and Implications | 129 |
| 5.4.1 | Approach: Engineering versus Science | 129 |
| 5.4.2 | A Control Paradigm | 130 |
| 5.5 | Cell Replication..... | 131 |
| 5.6 | Other Factors in the Cell Cycle | 144 |
| 5.6.1 | Ubiquitination | 146 |
| 5.6.2 | Kinetics of Cell Cycles | 149 |
| 5.7 | Summary | 149 |
| 6 | Intracellular Pathways..... | 150 |
| 6.1 | Pathway Overview | 150 |
| 6.1.1 | Global Cancer Pathway Models..... | 150 |
| 6.1.2 | The Elements | 152 |
| 6.1.3 | Specific Pathway Elements..... | 155 |
| 6.1.4 | Gene Specificity and Frequency | 156 |
| 6.2 | Some Specific Genes..... | 159 |
| 6.2.1 | Genes and HGPIN..... | 160 |
| 6.2.2 | PIA | 161 |
| 6.2.3 | PIN | 161 |
| 6.3 | HGPIN Genes..... | 165 |
| 6.3.1 | GSTP1 | 165 |
| 6.3.2 | CDKNA1B:p27..... | 167 |
| 6.4 | Generic Overview | 169 |
| 6.5 | The Single Cell..... | 174 |
| 6.6 | Putative Genes..... | 175 |
| 6.7 | Cell Surface Ligands and Receptors | 178 |
| 6.7.1 | Receptors..... | 178 |
| 6.7.2 | Ligands..... | 179 |
| 6.7.3 | Other Receptors | 187 |
| 6.8 | Pathway elements | 188 |
| 6.8.1 | Akt..... | 188 |
| 6.8.2 | PI3K | 191 |
| 6.8.3 | RAF..... | 192 |
| 6.8.4 | PTEN..... | 194 |
| 6.8.5 | Cyclin D | 197 |

| | | |
|--------|---|-----|
| 6.8.6 | SMAD4..... | 198 |
| 6.8.7 | SPP1..... | 201 |
| 6.8.8 | FOXO..... | 202 |
| 6.8.9 | mTOR..... | 205 |
| 6.8.10 | LKB1..... | 210 |
| 6.8.11 | PREX2..... | 217 |
| 6.8.12 | PTEN..... | 221 |
| 6.8.13 | EGFR, ERBB and Neu..... | 223 |
| 6.8.14 | AR..... | 225 |
| 6.8.15 | GRP78..... | 230 |
| 6.8.16 | TPMRSS2-ERG..... | 233 |
| 6.8.17 | NKX 3.1..... | 239 |
| 6.8.18 | Pleckstrin Homology..... | 241 |
| 6.8.19 | Crosstalk..... | 249 |
| 6.9 | Transcription Elements..... | 251 |
| 6.9.1 | cMYC..... | 251 |
| 6.9.2 | CDK..... | 253 |
| 6.9.3 | MITF..... | 253 |
| 6.9.4 | NF-κB..... | 259 |
| 6.10 | Pathways and Immune Response..... | 263 |
| 6.10.1 | Aberrant Pathway Control..... | 263 |
| 6.10.2 | B-RAF control..... | 265 |
| 6.10.3 | Immunological Techniques..... | 267 |
| 6.10.4 | Considerations..... | 268 |
| 6.11 | Ubiquitination..... | 269 |
| 6.12 | Conclusion..... | 269 |
| 6.13 | Appendix A: List of Genes Classified by Function..... | 271 |
| 6.14 | Appendix B: PH Genes and Locations..... | 286 |
| 7 | Prostate Stem Cells..... | 293 |
| 7.1 | The Stem Cell Paradigm..... | 296 |
| 7.2 | Progression and Regression..... | 306 |
| 7.3 | Stem Cell Complexity..... | 309 |
| 7.4 | PCa Stem Cell Recognition..... | 313 |
| 7.5 | Summary and Issues..... | 320 |

| | | |
|-------|--|-----|
| 8 | Epigenetic Factors..... | 321 |
| 8.1 | miRNA | 321 |
| 8.2 | siRNA and miRNA | 323 |
| 8.3 | Dynamics of miRNA..... | 323 |
| 8.4 | miRNA and Cancer | 324 |
| 8.5 | miRNA and Stem Cells | 327 |
| 8.6 | Methylation | 328 |
| 9 | Enzymatic Reactions and Pathways..... | 334 |
| 9.1 | Example Reaction | 334 |
| 9.2 | PCa Reaction Models: A Subset | 340 |
| 9.3 | HGPIN and GSTP1 | 340 |
| 9.4 | Total Pathways | 340 |
| 9.5 | Specific Pathways | 341 |
| 9.6 | Reaction Kinetics | 343 |
| 9.6.1 | Enzyme Reactions..... | 343 |
| 9.6.2 | Michaelis- Menten Model..... | 344 |
| 9.6.3 | Basic Kinetics and Reactions..... | 350 |
| 9.6.4 | Sigmoidal Reaction..... | 350 |
| 9.6.5 | Synthesis and Degradation..... | 351 |
| 9.6.6 | Negative Feedback..... | 351 |
| 9.6.7 | Activator Inhibitor | 352 |
| 9.6.8 | Substrate Depletion..... | 353 |
| 10 | Prognostic Markers | 356 |
| 10.1 | Recent Finding..... | 357 |
| 10.2 | Summary of Prognostic Gene Markers | 359 |
| 10.3 | TIMP-1 | 362 |
| 10.4 | ABL2 | 363 |
| 10.5 | SEMA4D | 364 |
| 10.6 | ITGAL | 368 |
| 10.7 | CDKN1A | 369 |
| 10.8 | C1QA..... | 372 |
| 10.9 | Observations | 373 |
| 11 | Other Prostate Cancer Issues | 376 |
| 11.1 | PCA3 and Prostate Cancer | 376 |

| | | |
|---------|---|-----|
| 11.2 | Homeobox | 379 |
| 11.3 | Wnt and TERT Signaling | 385 |
| 11.4 | Bad Cells Using Good Cells: Metastasis | 386 |
| 11.5 | Cancer Cells and the Environment | 388 |
| 11.6 | Stop and Go Genes | 388 |
| 11.7 | CNV and Prostate Cancer | 391 |
| 11.8 | Genetic Screening for Prostate Cancer | 395 |
| 11.9 | Summary | 398 |
| 12 | Pathway Models, Validation and Controls | 399 |
| 12.1 | Cell Growth and Its Metabolic Control | 401 |
| 12.2 | Pathway Dynamics | 402 |
| 12.3 | Pathways, Gene Expression Measurement, Metastasis | 404 |
| 12.3.1 | Dana Farber Study | 405 |
| 12.3.2 | PTEN Suppression | 406 |
| 12.3.3 | Cyclin D | 408 |
| 12.3.4 | SMAD4 | 409 |
| 12.3.5 | SPP1 | 412 |
| 12.3.6 | Analysis | 413 |
| 12.3.7 | Gene Tables | 416 |
| 12.12 | Pathway Models | 424 |
| 12.12.1 | Boolean Networks | 424 |
| 12.12.2 | Bayesian Networks | 427 |
| 12.3.8 | Bayesian Analysis | 428 |
| 12.3.9 | Microarrays | 431 |
| 12.3.10 | Bayesian Network Summary | 434 |
| 12.12.3 | State Dynamic Networks | 435 |
| 12.13 | Identification, Regression and its Drivers | 440 |
| 12.13.1 | Identification | 440 |
| 12.4 | Measurements | 446 |
| 12.5 | Inversion Approaches | 448 |
| 12.5.1 | CIE Approach | 449 |
| 12.5.2 | Newton Steepest Descent | 450 |
| 12.5.3 | Kalman Filter | 451 |
| 12.5.4 | The Matched Filter Approach | 454 |

| | | |
|--------|--|-----|
| 13 | Total Cellular Dynamics | 458 |
| 13.1 | Assumptions | 462 |
| 13.2 | Total Cellular Dynamics Models..... | 463 |
| 13.2.1 | Turing Tessellation | 466 |
| 13.2.2 | Determining the Coefficients | 467 |
| 13.2.3 | Implications..... | 468 |
| 13.3 | Stochastic Models..... | 470 |
| 13.4 | Stability..... | 471 |
| 13.5 | Controllable and Observable | 472 |
| 13.6 | Controllability..... | 472 |
| 13.7 | Observability | 473 |
| 13.8 | Summary..... | 473 |
| 14 | Conclusion | 475 |
| 14.1 | Preamble | 475 |
| 14.2 | HGPIN and a Precursor | 476 |
| 14.3 | Screening for Prostate Cancer: The Task Force Report | 478 |
| 14.4 | PSA Effectiveness and Screening Interval Time..... | 486 |
| 14.5 | Cancer Metabolism: An Example of Complexity | 497 |
| 14.6 | Cancer and The Immune System..... | 499 |
| 14.7 | Summary..... | 500 |
| 15 | References..... | 502 |

1 INTRODUCTION

This is a work on a systems view of a specific cancer, prostate cancer, or PCa. This is a highly complex cancer and is also one of the most common in men. It is also one which has caused a great deal of debate because it is often indolent but when not it is a deadly killer. Thus by saying that a person has prostate cancer is not an adequate diagnosis; one must delve much deeper to understand what of the many variations that patient has. The changes in health care however suggest that simple all-encompassing answers are ought so that “rule” may be applied to control costs. Thus the development of Government centered and controlled Comparative Effectiveness Research (“CER”) and its ensuing guidelines, leaves little room to understanding the subtlety of this disease. As we have seen in the last forty years with our understanding of leukemias, prostate cancer has many faces.

The “engineering” approach we take herein differs from a medical or scientific approach in several ways. The Medical approach general focuses on diagnosis and treatment. What is necessary to diagnosis at cell or even gene level, and then using those diagnostic elements how does one lay out a treatment path? Treatment is chosen based generally upon proven efficacy. Physicians are generally reluctant to try treatments whose efficacy has not been established for legal as well as ethical reasons.

In contrast the scientific approach is one focused on establishing the understanding of basic facts, cells elements, genes. gene influences, presence of gene expressions in various disease states. It is the very essence of new discoveries that drive the scientific approach.

In contrast the engineering approach is somewhat in the middle ground. There is an emphasis on using and integrating proven knowledge and in selecting those elements which are useful and neglecting until proven otherwise elements which are more cumbersome. The engineering approach looks at synthesizing the elements into a holistic systematic whole. The engineering approach looks to build verifiable models and from those models seek ways to change outcomes, control the process, and effect possible improvements or cures. In many ways the patient gets in the way as an overly complex and multifaceted presenter of the disease state. Thus as the physician must deal with the entire person, and the scientists deals with the cell with almost total disregard of where it came from, the engineering approach can have tension regarding the ultimate carrier of the process being controlled.

We have divided this work into three sections. Each focuses on a different aspect but they are in effect a totality of the elements of the systems approach.

1.1 SECTION ONE: PSA AND PROSTATE CANCER

In the first section we examine in some detail the PSA controversy. PSA can be used and has been used as a marker for prostate cancer, PCa. However in the past few years there has been a massive amount of controversy regarding this use driven mostly by the exploding costs of health care. A similar but smaller controversy has arisen in the area of breast cancer but unlike breast cancer men who have PCa are now being asked to be treated with “watchful waiting” which is a euphemism for doing nothing. That is the low cost approach and yet it may work in many cases

whereas for those in which it does not the results are agonizingly painful and deadly. Thus the question we examine in this section is the issue of PSA, its effectiveness, and the politics around which it is examined. In this section we look a bit further into the PSA issue but introduce certain cellular metrics and temporal statistics. Cancer like any disease is a process, a slow at time process but if measurements are made we can then utilize those measurements and ascertain better diagnostic and prognostic results. We do that in this section. It introduces the dynamics of the disease.

1.2 PROSTATE CANCER AND GENETIC PATHWAYS

In this section we enter the details of the genetic makeup of the prostate cells and specifically those genes which putatively are involved in PCa. We first review the PCa development through various stages and then focus on the major genes involved. We then present the current pathways for each of the genes and from this we develop a systems model for PCa. We examine the approaches other are also taking in this area and specifically we discuss the need for understanding pathways rather than just presenting genes as-is. We examine various methodologies for building both steady state and dynamic pathway models.

1.3 PROSTATE CANCER VIA A SYSTEMS APPROACH

This area examines PCa as a system of networks, networks of genes, genes which are ligands, receptors, pathway elements and nucleic elements. It creates temporal models in an intracellular manner and we then extend it to a fully distributed manner. We examine what goes wrong within a cell and then what goes wrong across the total body. In this examination we explore two elements in a systems context; first, we look at the ability to “observe” the system and in that context identify the key system parameters, second, we examine the ability to “control” the system which from an engineering context means drive it to a specific state, or in the medical context control the disease state. We present methodologies; we do not present definitive conclusions.

1.4 DEFICIENCIES

There are certain deficiencies we have in this effort. Let us look at a few:

1. PCa is Complex: We would argue that PCa is not just one disease. There may be several different genetic presentations which are reflected in the histological and pathological presentation. Thus PCa may be more akin to leukemias as we understood those forty years ago. There may be many different subclasses identifiable only through complicated genetic markers. Thus there may therefore be multiple models for the disease.
2. Exogenous Factors: There are many exogenous factors which have come to the fore. These are the methylation effects and the impact of miRNAs. These are most likely just two of many which will impact pathway dynamics. If they are dominant or recessive we still do not know.
3. Intracellular and Intercellular Pathways: The pathway models generally focus on a single cell. Yet there are intercellular communications which may play a significant role. We have examined

that in other species and find the intercellular effect must not be neglected. In the approach herein we have neglected them deliberately. The reason, we have little or no science on them at this stage.

4. Cancer Stem Cells: The cancer stem cell hypothesis is another piece of sand in the gears. The cancer stem cell, CSC, is basically the theory that only a few of the cancer cells are pluripotent and involved in growth. If so we need to understand the dynamics of that cell apart from the others. Thus the techniques we use and propose assume that the microarray data is used as if the cells all acted the same. Yet what we may truly need is a set of cells which can allow us to separate the CSC from the others and examine them alone.

1.5 THE PROSTATE CANCER DEBATE

We mix in our presentation considerable discussion of PCa and the PSA debate. This debate is reflective of the change occurring in medicine, namely control of costs by implicitly rationing care. Unlike a more classic medical text, or even an engineering text, we examine these issues in some detail because they go to the heart of an overall systems view. They drive new examinations of what appear to be medically accepted results. For example there were recent studies in NEJM which we examine in detail. We argue that the results are not reflective of reality, and we propose alternatives. We do not contend that we have any clinical basis for these alternatives, yet they evolve from an engineering mindset, not a clinical one. Furthermore our views are holistic in form, we examine the human in toto.

2 PSA AND ITS IMPLICATIONS

This Chapter deals with the use of one specific marker for prostate cancer. It looks at PSA, the prostate specific antigen, and it develops quantitative methods to use this antigen and evaluates others which are useful in the application of PSA. There has been a great deal of discussion and confusion regarding this test as well as a great deal of misstatements on the part of many researchers. The recent publication of the of the American and European studies in NEJM on the use of PSA and their conclusions that the monitoring of PSA did not affect any change in outcomes we believe is grossly in error. We make the argument herein that PSA is a useful tool albeit a limited one.

Now this Chapter places the initial emphasis of the book on PSA. PSA is really the tail that seems to be wagging the dog. Namely physicians generally perform the test and it may or may not be diagnostic. It must be taken in context of many other factors and it is hardly prognostic unless at an excessive level ab initio. Rather than starting with genes, which frankly is what we are concerned about, one must first understand the context in which we will make our arguments, a highly politicized context. In addition with the passing of the ACA and with Government panels formed to cut costs, the PSA becomes the stalking horse for PCa. Thus we start with it even though it is the result of what we plan to discuss.

PSA is excreted by luminal cells in the prostate gland and is absorbed into the blood stream. The more such cells the higher the concentration of PSA and thus one could conceive, rightly so, that monitoring PSA is in effect monitoring the cells in the prostate. Exceptionally high growth of the prostate cells is often associated cancer. Thus PSA may be a harbinger of cancer and if it exceeds certain levels further studies should be performed.

However PSA alone is not the sine qua non in this process. One must look at this in the context of the patient as a totality. The patient family history oftentimes trumps all since a first degree relative with an aggressive form of prostate cancer is more of a concern than just an elevated PSA. Prostate size in and of itself is a cause just because there are more cells. The change of PSA over time is also a significant factor. All too often the physician has little if any notion of that change. We believe that temporal factors of a patient are key to effective medical records, especially electronic medical records and this factor seem to play second seat to other administrative factors. The PSA velocity is a major factor ascertainable only by having the access to all records.

Finally and most importantly is the PSA assay itself. Measuring of PSA may vary by 25-50% from assay to assay. This is a clinical problem when seeking to ascertain such factors as PSA velocity.

We will now look at PSA and its clinical use as well as looking at the methods and techniques by which various researchers have tried to reach the conclusions that they have. The work of Punglia and others have demonstrated, in 2003, the thresholds for PSA tests vary dramatically from the young to the old. What has not been analyzed has been using PSA as a measure for ascertaining aggressive versus indolent prostate cancer, PCa. This has yet to be determined. We know the pathways but even using various stains to ascertain the presence or absence of certain

proteins in the pathways has not evolved into a useful and predictable set of tests for aggressive types. That type of test will be the sine qua non for monitoring PCa.

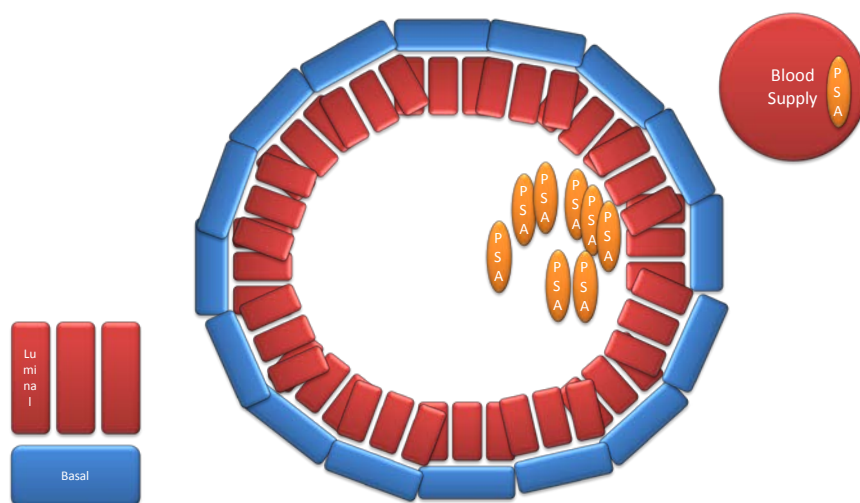
2.1 PSA FUNCTION

Before detailing the cellular level of the pathology it is worth while discussing the PSA issue and the controversies related thereto.

The normal prostate is a 40 cc globe like gland just below the bladder and surrounding the urethra. It is composed of 35-50 small glands and between the glands is a stroma composed of nerves, muscles, and blood supplies, with some other connective tissues. A typical gland is shown below along with an adjacent blood flow.

The following Figure graphically depicts the gland in the prostate and the PSA released mostly into the lumen of the gland but a small percent gets released into the blood supply.

Normal Prostate Gland and PSA



PSA, prostate specific antigen, is a gene product of chromosome 19¹. The PSA gene is androgen regulated. PSA is synthesized in the epithelial cells. It is secreted into the lumen of the prostate gland ducts and works its way into the serum most likely by diffusion. PSA tends to increase with hypertrophy and PCa. This most likely is due to cell proliferation and thus a larger base of excretion of PSA into the lumen. There does not however seem to be any studies relating serum PSA to prostate size, volume. A normal prostate is about 40 cc in volume and large prostates say of 60 cc may have more epithelial cells and thus putatively a larger PSA in the serum, however there does not appear to be evidence supporting this conjecture.

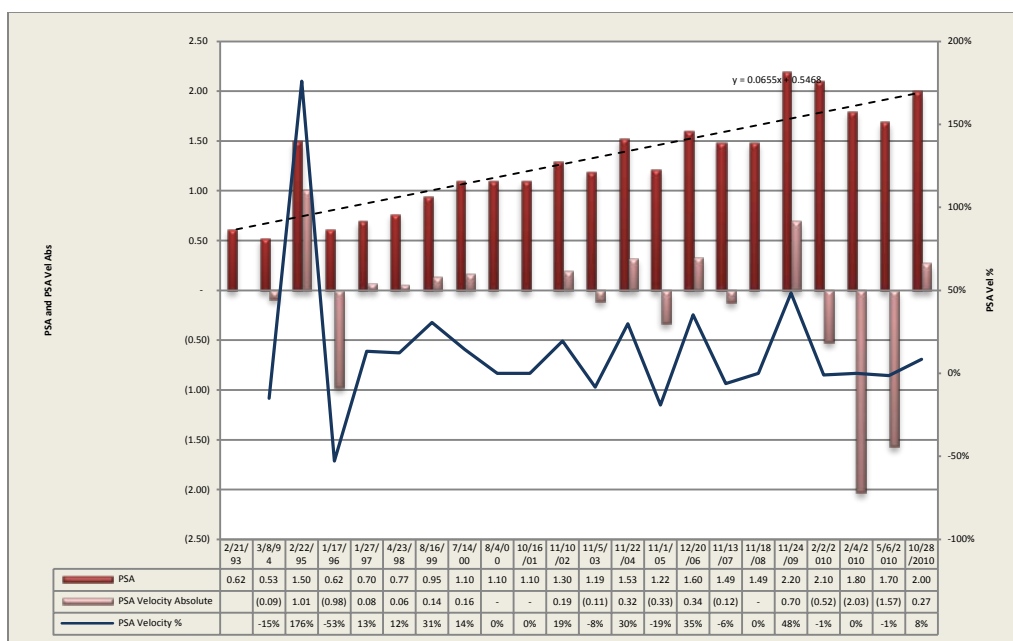
¹ See Kantoff, Prostate, p 213.

Most serum PSA is bound to proteins. Some is unbound and thus free. Thus, the Percent Free PSA is often also measured. PSA released from cancer cells however is often not processed by intracellular proteolytic chains and thus is not free. High percent free is often a sign of no malignancy².

PSA velocity is another measure of malignancy potential. The definition of PSA velocity is the three sample average of PSA change per year or percent change per year. That is, we take three time samples, and then calculate two velocities, from the second less first, and the third less second, and annualize each and take the average. If the velocity exceed 0.75 we have a threshold which requires examination³.

2.1.1 PSA

We can now look at a typical PSA history. We show below a 20 year PSA history of a patient where we also show velocity as well as PSA change. The first problem we would have here is that there are most likely a dozen different assays so that any comparisons are difficult to make due to the fact that each may be from a different assay. Namely the assays do show material variation from one another. Secondly, we have a 20 year temporal change in any one assay as well, thus any consistent baseline is often in question. Specifically we have intra and inter assay bias and noise.



Thus looking at the above we see a 4 fold increase in 20 years. This is for a male from 50-70 years of age. The change in the prostate during that period may be significant. It may grow in size and thus have increasing cells, it may have PIN, prostatic intraepithelial neoplasia, and also

² Su, Prostate, p 5.

³ Su, prostate. p 5.

have more luminal cells, or it may have a low grade PCa. Thus looking at this patient one must ask what to do next? It will of course depend on family history more than the PSA changes.

2.1.2 PSA Velocity

In a paper by Carter et al the authors provide an excellent review and analysis of the use of PSA velocity. As the authors state, a driver for this study is:

Recently, D'Amico et al. showed that, when compared with men with a PSA velocity of 2.0 ng/mL per year or less in the year before diagnosis, men with a PSA velocity above 2.0 ng/mL per year were at an increased risk of prostate cancer death after surgical treatment. An unanswered question is whether a lower PSA velocity could identify those men with life-threatening prostate cancer during a window of curability.

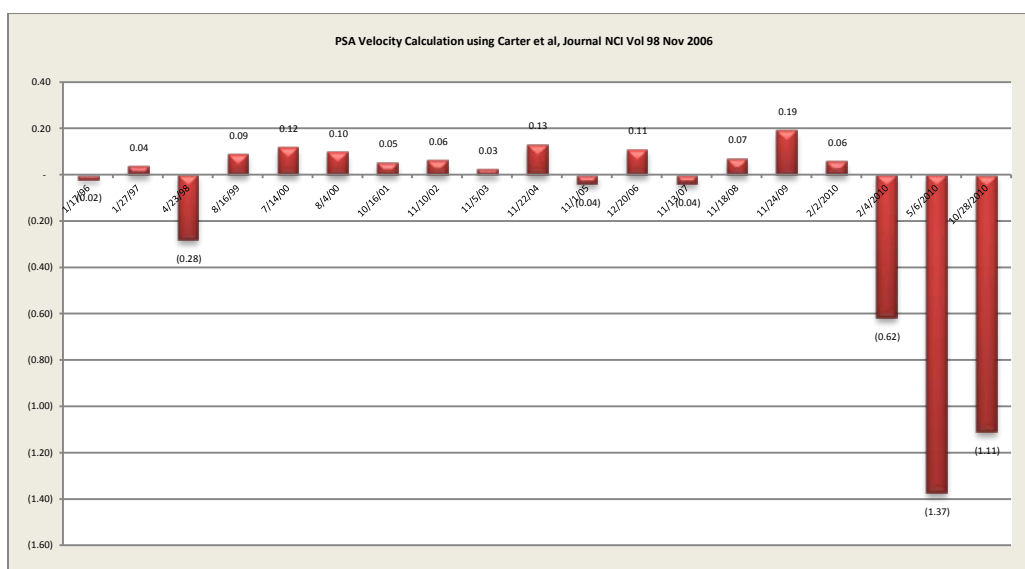
This PSA velocity is a significant factor. As defined by Carter et al:

PSA velocity in ng/mL per year was calculated for each subject (n = 788) as the running average of the rate of change over three consecutive visits (the index visit and the two preceding visits), when more than two PSA measurements were available (5), or as the simple rate of change, if only two measurements were available.

Or we can use the following:

$$V_{PSA}(k) = \frac{\sum_{n=k-3}^k \frac{PSA(n) - PSA(n-1)}{Date(n) - Date(n-1)}}{3}$$

We have used this formula on the data above and have shown the velocity where we use units in years. The negative values are driven by a single poor PSA reading. One must be careful in performing this analysis to include consistent assays. That is often the problem.



The results by Carter et al are:

PSA velocity measured 10 – 15 years before diagnosis (when most men had PSA levels below 4.0 ng/mL) was associated with cancer specific survival 25 years later;

survival was 92% (95% confidence interval [CI] = 84% to 96%) among men with PSA velocity of 0.35 ng/mL per year or less

and 54% (95% CI = 15% to 82%) among men with PSA velocity above 0.35 ng/mL per year ($P < .001$).

Furthermore, men with PSA velocity above 0.35 ng/mL per year had a higher relative risk of prostate cancer death than men with PSA velocity of 0.35 ng/mL per year or less (RR = 4.7, 95% CI = 1.3 to 16.5; $P = .02$);

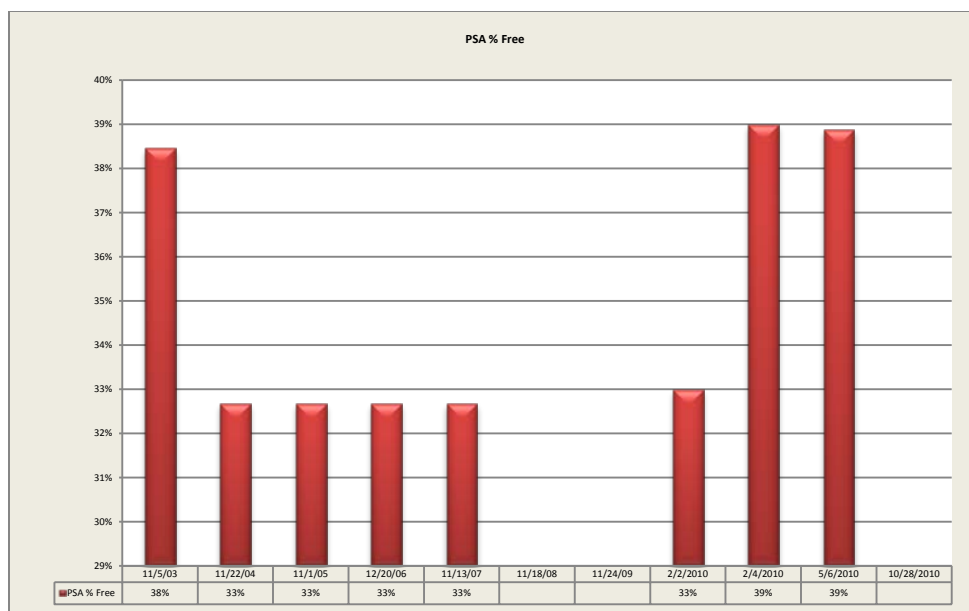
the rates per 100 000 person-years were 1240 for men with a PSA velocity above 0.35 ng/mL per year and 140 for men with a PSA velocity of 0.35 ng/mL per year or less.

Thus in looking at the above patient we should conclude with Carter that even if PCa is discovered it should have a reasonably good chance of survival. Yet again the issue is always one of assay consistency.

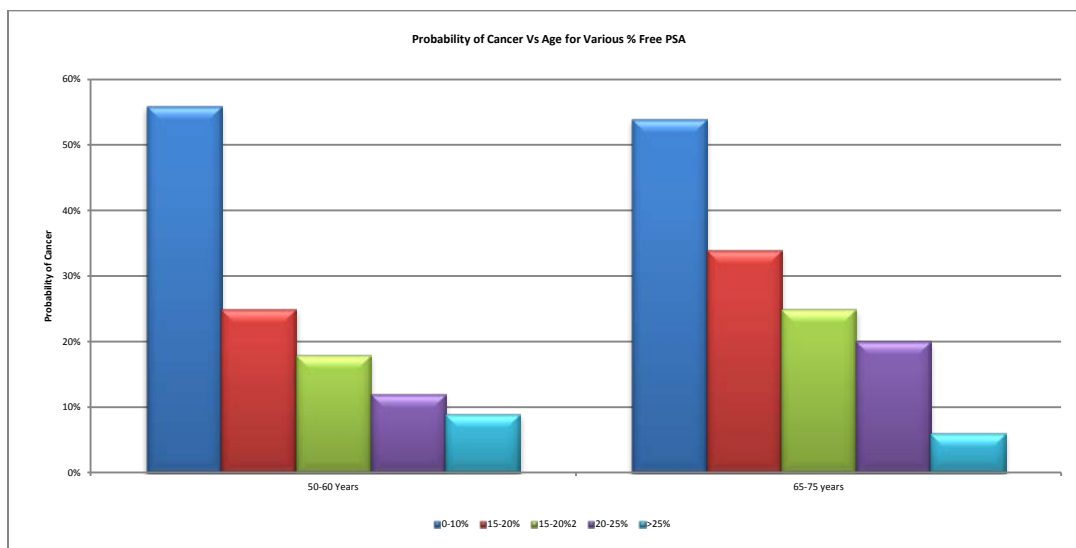
2.1.3 Percent Free PSA

The percent free PSA is a measure of the PSA generated by benign luminal cells which is unbound to proteins in the circulation. The majority of PSA in the blood stream is bound to proteins, primarily α -antichymotrypsin. The remaining amount, from about 5% to 35%, is free. PSA released from cancer cells is generally bound and not free. Thus the increase in PSA with a concomitant reduction in percent free is an implication of PCa. On the other hand, if PSA slightly rises and free PSA also rises, or stays at peak, then one would suspect a benign process of hyperplasia or benign or non-malignant neoplasia. As we progress to PCa, the luminal cells which are malignant clones do not have free PSA and thus the percent free drops.

In the case of the patient we have been examining we see a percent free in excess of 39% which appears to indicate no malignancy.



Thus, the above Percent Free PSA appears to be benign. Again we also must note the change in values may be driven more by the assay than any underlying process. The chart below is modified from Yang and the data taken from Catalona et al. It shows that the higher the percent free the lower the risk of cancer.



2.2 THE PSA CONTROVERSY

The use of PSA has become quite controversial over the past few years and especially as the new health care laws have been mandated by the Democratic Congress. The main putative issue is the subsequent biopsies required and their costs as well as the resulting prostatectomies and their morbidities as well as costs, given the putative prevalence of indolent PCa. Namely there are groups who argue that PCa is generally a benign disease and that with death as an end point, the actions resulting from PSA measurements are often lacking in changing the end point, namely men die at the same rate whether treated or not.

Professor Ablin, the researcher who allegedly discovered the PSA antigen which is used in testing for prostate cancer, PCa, wrote a scathing editorial in the NY Times this decrying the test and its implications⁴. He starts by stating:

The test's popularity has led to a hugely expensive public health disaster. It's an issue I am painfully familiar with — I discovered P.S.A. in 1970. As Congress searches for ways to cut costs in our health care system, a significant savings could come from changing the way the antigen is used to screen for prostate cancer.

Americans spend an enormous amount testing for prostate cancer. The annual bill for P.S.A. screening is at least \$3 billion, with much of it paid for by Medicare and the Veterans Administration.

There is significant disagreement here. That the PSA test alone has some problems, which is well recognized. Yet this test alone, as a single measurement upon which to act dramatically, was never intended to be used that way. Thus the whole basis for his argument lacks any substantial merit. We will make the argument as follows.

1. PSA by itself as a onetime test with a threshold of 4.0 as applied to all men does not significantly reduce mortality. This is a true fact. The Professor states:

The medical community is slowly turning against P.S.A. screening. Last year, The New England Journal of Medicine published results from the two largest studies of the screening procedure, one in Europe and one in the United States. The results from the American study show that over a period of 7 to 10 years, screening did not reduce the death rate in men 55 and over.

The European study showed a small decline in death rates, but also found that 48 men would need to be treated to save one life. That's 47 men who, in all likelihood, can no longer function sexually or stay out of the bathroom for long.

As we will demonstrate, these studies used the 4.0 level as the benchmark and the European study had long periods between testing and the US study did two year testing and again applied 4.0 for all.

None of the studies recognized the newer research that said that 2.0 was the threshold for those under 60 and that velocity was a major component to be added. Velocity, the averaged change in PSA per year, is recognized as a major factor and that if the velocity exceeds 0.75 per year for men over 65 and with a stable PSA over 4.0 then the sensitivity and specificity rises appreciably. Second for men under 60 or with a baseline long term PSA under 2.0, if the velocity exceeds 0.25 the sensitivity and specificity also is quite high.

Also we know that free PSA and % Free PSA are further indicators of PCa, since the PCa cells bind the free PSA whereas the normal acinar cells do not.

⁴ <http://www.nytimes.com/2010/03/10/opinion/10Ablin.html>

Finally, family history is critical. It falls into three categories; no PCa, PCa of an indolent form, and PCa of a virulent form.

Thus if one has no PCa in one's family then most likely you have a lesser chance of having a virulent PCa. If your family history is of indolent forms then there is a good chance you too with have that form. If your family history is of a virulent form then you too may most likely have that form. What is a virulent form, we have seen that form many times. The PSA went from 4 to 40 in two years and 40 to death in two years! Why did that happen, well we do not yet fully know the dynamics of the cancer pathways, we do know that PTEN and its pathway were knocked out at some point and off it went.

Using a Bayes methodology, we really want to measure the following probability:

P[PCa| PSA, PSA Velocity, Percent Free, Percent Free Velocity, Family History]

Then given the a priori data we can determine an a posteriori probability and act accordingly. Professor Albin appears to neglect all of these facts. Albin continues his exhortation:

So why is it still used? Because drug companies continue peddling the tests and advocacy groups push “prostate cancer awareness” by encouraging men to get screened. Shamefully, the American Urological Association still recommends screening, while the National Cancer Institute is vague on the issue, stating that the evidence is unclear.

The federal panel empowered to evaluate cancer screening tests, the Preventive Services Task Force, recently recommended against P.S.A. screening for men aged 75 or older. But the group has still not made a recommendation either way for younger men.

Prostate-specific antigen testing does have a place. After treatment for prostate cancer, for instance, a rapidly rising score indicates a return of the disease. And men with a family history of prostate cancer should probably get tested regularly. If their score starts skyrocketing, it could mean cancer.

The test, when combined with other variables has been shown to have merit. Yet one of the factors is the patient's history, the long term PSA data, not a single PSA measurement. One of the problems with a single PSA measurement is that there is a +/- 50% variation in PSA measurements. The PSA may vary from say 1.5 to 1.8 to 2.1, to 2.1 in the same person but using differing assays. That, in itself, would set off alarms. Yet, if there were a 20 year history then one could better determine the velocity and watch for results and not jump to surgery. Albin seems to reject the volume of clinical data with his position. Yet Albin's position is all too common and one wonders why.

One can also look at more Facts. For example, prostate biopsies, using the classic sextant or 6 core forms, have been notoriously poor in detecting cancer. In addition the biopsy cannot as current performed determine indolent versus virulent forms, that is a genetic marker issue. One could do an assay on the cells for PTEN marker presence but that is still an experimental procedure. One could use the PCA3 test which determines Gleason 7 or greater with reasonable

specificity and sensitivity but that is only a recent development and by the time one gets to Gleason 7 one may have a PCa which will have positive margins after prostatectomy.

One would like to get PCa at Gleason 5 or 6 with negative margins. This often means more cores. Thus for say a 40 cc prostate one needs 12 to 14 cores, and yet one may still have a 20% or greater chance of missing a cancer. In a larger prostate, say 60 cc one may need 20 cores and yet still have an almost 20% chance of detecting a PCa on the next biopsy say 6 months later. The problem is that we do not have the genetic tools to detect PCa, and in fact almost all Cancers, at the earliest a stage. The problem with PCa is that we do not know the indolent from the virulent from even at biopsy.

Is the answer as Albin argues seems to be to just abandon the testing. Death from PCa is not a pretty picture, it is akin to breast cancer, especially with mets to the bone. Mets to bones, collapse of the spine, result in disseminated intravascular coagulation, and is not a pretty picture.

The House Oversight Committee has held hearings on prostate cancer and testing and their intent seems to influence CMS to reduce the screening⁵. The American Cancer Society issued new guidelines for screening and they seem to retain PSA screening⁶. NIH recounts the ACS guidelines as follows⁷:

In new guidelines released ... the society (the ACS) says that men who choose to be tested should get an annual screening if their level of prostate-specific antigen, or PSA, is 2.5 nanograms per milliliter (ng/mL) or higher. But men whose PSA is under that threshold can be safely screened every two years. Men with a PSA level of 4.0 ng/mL or higher should consider getting further evaluation, such as a biopsy. Previous guidelines had suggested that men with a PSA of less than 4.0 ng/mL should be screened annually.

While the cancer society does not recommend screening for anyone -- even men at risk -- it does offer suggested intervals for screening if men choose to be tested.

The ACS specifically states:

Studies are being done to try to figure out if early detection tests for prostate cancer in large groups of men will lower the prostate cancer death rate. The most recent results from 2 large studies were conflicting, and didn't offer clear answers.

Early results from a study done in the United States found that annual screening with PSA and DRE did detect more prostate cancers, but this screening did not lower the death rate from prostate cancer. A European study did find a lower risk of death from prostate cancer with PSA screening (done about once every 4 years), but the researchers estimated that about 1,400 men

⁵ http://oversight.house.gov/index.php?option=com_jcalpro&Itemid=1&extmode=view&extid=126

⁶ <http://caonline.amcancersoc.org/cgi/content/full/caac.20066v1>

⁷ http://www.nlm.nih.gov/medlineplus/news/fullstory_103229.html

would need to be screened (and 48 treated) in order to prevent one death from prostate cancer. Neither of these studies has shown that PSA screening helps men live longer (lowered the overall death rate).

The statement is wrong about the two studies released in 2009 and we will detail the analysis later in this section. However to summarize our objections to the two studies, they both used the 4.0 PSA level and the testing was sporadic at best, failing to do annual tests, lacking % Free PSA data, and especially failing in any meaningful measurement of PSA velocity. The answer is that mortality will most likely not change if one waits until a 4.0 is reached in many sub-groups. The set point was reduced to 2.0 in the Punglia et al work we discuss herein as data was obtained but the trial never tested the lower level thus by leaving it at 4.0 they allowed the cancers to grow to a terminal stage. In addition the time between testing was excessive, in our analysis annually at least should be done, and in addition a ten year benign time horizon is also essential.

The ACS continues:

Prostate cancer tends to be a slow growing cancer, so the effects of screening in these studies may become clearer in the coming years. Both of these studies are being continued to see if longer follow-up will give clearer results.

This is also in error. Prostate cancer falls in two categories; slow growing or indolent and this represents about 90% of all such cancers and fast growing deadly type which kills in 4 years or less. The recommendation of the ACS could be a death verdict for the men in the latter category. The problem is that we do not know genetically how to determine this category.

For example, we now know that two factors, percent free PSA and PSA velocity are major factors and not just PSA. Percent free is a measure of the percent of cells which are functioning normally, albeit they may be PIN cells, prostatic intraepithelial neoplasia, high grade, HGPIN, which may be a precursor to prostate cancer. HG PIN must be monitored by biopsy on a schedule of three to four times a year! Not ignored. Velocity is critical since it is a reasonable measure for the growth of cells. Also a measure for both PIN and prostate cancer.

We know that even a biopsy can at best be 10-25% in error. A 20 core biopsy can still miss cancer with a 10% probability. In addition a second biopsy using 14 or more cores may find cancer 25% of the time or more on a second testing!

The aggressive prostate cancer can kill a man in less than 4 years! Do we want that risk? If you are in that group I would think not. What further helps, family history. If you have had a first degree relative who died in a short period then it is highly likely that you have inherited the genetic errors that allow rapid growth, namely the elimination of the PTEN gene and thus metastasis.

The ACS also states:

Because of these complex issues, the American Cancer Society recommends that doctors more heavily involve patients in the decision of whether to get screened for prostate cancer. To that end, ACS's revised guidelines recommend that men use decision-making tools to help them make

an informed choice about testing. The guidelines also identify the type of information that should be given to men to help them make this decision.

The problem is how do you involve a man if the physician has no understanding and in fact is confused given the literature. Biopsy is not a gold standard, it may be a silver or bronze. If the biopsy yields a Gleason 6, rarely less since most pathologists will grade 3+3 yielding Gleason 6, and almost never grade a 1 nor even a 2, then one still does not know the genetic makeup, the true determinant. In fact most physicians do not understand the genetic factors, including many urologists. Thus in many ways it is the blind leading the blind, and the ACS has done nothing more than put stumbling blocks in the way. Further by testifying before Congress they have done men a disservice. Yet it does reduce Medicare costs, we just let those old folks die, and yes many young ones too.

2.3 THE PSA DEBATE

The focal point of many of the arguments over the usefulness of PSA measurements has been the publication in NEJM of two studies. We summarize them as follows and then consider them in detail. In summary we believe that the studies albeit initially designed well given what was known at the time, were, at their conclusion, asking the wrong question and failed to do what was initially intended. Simply stated the studies asked if using 4.0 as a cutoff for PSA saved lives. The answer was no, but the studies were defective in many ways as we shall describe and in addition they also asked the wrong question. They should have asked a question as follows:

“What level of PSA and PSA related measurements, such as % Free PSA, measured over time, and using age and other related factors, would result in a significant reduction of mortality?”

Namely it would be a more open ended question. We examine the studies as follows.

2.3.1 The American and European Studies

The New England Journal of Medicine published two studies on prostate cancer screening. Before presenting their results for analysis let me first show what the NY Times said. Their headline was: "Prostate Test Found to Save Few Lives"

First the NY Times author, Gina Kolata, states:

"The PSA test, which measures a protein released by prostate cells, does what it is supposed to do — indicates a cancer might be present, leading to biopsies to determine if there is a tumor. But it has been difficult to know whether finding prostate cancer early saves lives. Most of the cancers tend to grow very slowly and are never a threat and, with the faster-growing ones, even early diagnosis might be too late."

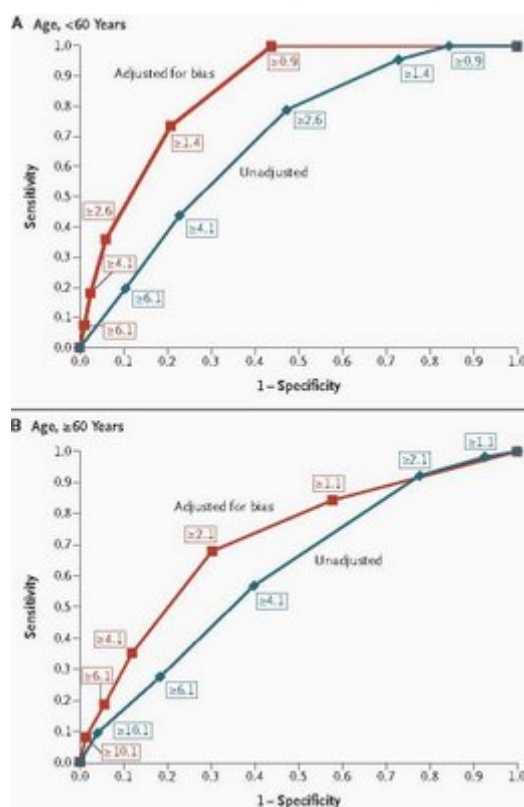
The PSA test is not just one test. It is not a black and white thing. It is a process that has evolved over time. There is not a good and bad PSA per se. Admittedly if you are 65 and have a PSA of 60 you are in some trouble. But as we now know a PSA of 2.1 when you are 50 is of concern.

But more critically the rate of change in PSA is almost diagnostic. Thus a 25% rate of increase per year should be followed up.

In July 2003 Punglia et al in the New England Journal of Medicine published a study which demonstrated that the then current set point for PSA missed many cancers. They stated:

"Adjusting for verification bias significantly increased the area under the ROC curve (i.e., the overall diagnostic performance) of the PSA test, as compared with an unadjusted analysis (0.86 vs. 0.69, $P<0.001$, for men less than 60 years of age; 0.72 vs. 0.62, $P=0.008$, for men 60 years of age or older). If the threshold PSA value for undergoing biopsy were set at 4.1 ng per milliliter, 82 percent of cancers in younger men and 65 percent of cancers in older men would be missed. A digital rectal examination that is abnormal but not suspicious for cancer does not affect the overall performance characteristics of the test....A lower threshold level of PSA for recommending prostate biopsy, particularly in younger men, may improve the clinical value of the PSA test."

They presented the following Figure:



The PSA test has been refined over the period of these studies, the PLCO Study, "Prostate, Lung, Colon, Ovary".

Now to issue two; let us assume that a biopsy is performed. If a Gleason score of 7 is noted then you best have some attention paid, even a 6 is a problem. You have cancer! It will grow. It may

very well kill you! That is if you do not die of something else. The problem is twofold; first, the doubling time of the cancer cells may be short, and second, the metastatic potential could be great. For Prostate cancer has the habit of metastasizing to the bones, especially the spine. Does one want to take that risk?

The European study states the following protocol:

*"We identified 182,000 men between the ages of 50 and 74 years through registries in seven European countries for inclusion in our study. The men were randomly assigned to a group that **was offered PSA screening at an average of once every 4 years** or to a control group that did not receive such screening. The predefined core age group for this study included 162,243 men between the ages of 55 and 69 years. The primary outcome was the rate of death from prostate cancer. Mortality follow-up was identical for the two study groups and ended on December 31, 2006..."*

The European trial is akin to a Fire House which uses an answering machine which it checks every three days to see if there is a fire. They then study the town with this Fire House and a town without a Fire House and discover that there is no difference in destroyed houses. Well one would perhaps think that having someone there to answer the phone when it rings and then immediately dispatching a fire engine would improve things. That is not what they apparently did. Also they did a test once every four years, we contend that annual is necessary due to the PCa doubling time.

Let us explain. PSA screening once every year, this is based upon a tumor doubling time of 3 months, a DRE and PSA are performed. If the PSA is measured as per Punglia statistic then we would use 2.6 for men under 60. Punglia states:

"These findings, as well as recent data from a randomized trial showing that prostate-cancer treatment improves disease-free survival, 28 indicate that reduction of the threshold PSA level at which biopsy is recommended to 2.6 ng per milliliter, at least in men under 60 years of age, may be reasonable."

Subsequent studies indicate that the added measurement of velocity or rate of change per year is also critical. Thus a 25% per year rate of change should be used as a way to seek an examination.

The American Group as published in NEJM provides the following results:

"From 1993 through 2001, we randomly assigned 76,693 men at 10 U.S. study centers to receive either annual screening (38,343 subjects) or usual care as the control (38,350 subjects). Men in the screening group were offered annual PSA testing for 6 years and digital rectal examination for 4 years. The subjects and health care providers received the results and decided on the type of follow-up evaluation. Usual care sometimes included screening, as some organizations have recommended."

The numbers of all cancers and deaths and causes of death were ascertained....In the screening group, rates of compliance were 85% for PSA testing and 86% for digital rectal examination."

Rates of screening in the control group increased from 40% in the first year to 52% in the sixth year for PSA testing and ranged from 41 to 46% for digital rectal examination. After 7 years of follow-up, the incidence of prostate cancer per 10,000 person-years was 116 (2820 cancers) in the screening group and 95 (2322 cancers) in the control group (rate ratio, 1.22; 95% confidence interval [CI], 1.16 to 1.29). The incidence of death per 10,000 person-years was 2.0 (50 deaths) in the screening group and 1.7 (44 deaths) in the control group (rate ratio, 1.13; 95% CI, 0.75 to 1.70)."

This American group was one with PSA at 4.0 and a second where PSA may or may not have been used as was a DRE. This is NOT a comparison of two distinct samples. The control group is a mix of anything and everything. Thus there are in my opinion two major faults;

First, the PSA numbers were set too high since we now know they should be set lower.

Second, the Control group was not the untested group as may be inferred, it was unlike the European study which alleges no treatment, and it was tested but just haphazardly.

Thus we have four groups:

Group 1 (American): PSA at 4.0 and DRE annually

Group 2: (American) PSA at 4.0 and DRE haphazardly

Group 3: (European) PSA at 4.0 but only once every 4 years

Group 4: (European) No screening

What is missing is what we now know to be the case. A PSA at 2.0 and an age dependent PSA with velocity measurements.

Thus our conclusion is that the Bayesian analysis, namely determining the probability of death given PSA measurements is or is not independent of the PSA measurement. We believe that the Bayesian approach of using screening at 2.0 under 60 and then testing and addressing a malignancy will reduce the a posteriori mortality. The data assessing that hypothesis appears to bear that out.

The NY Times headline is confusing, and frankly in error. The study proved at best that the specific screening protocol did not result in longer lives. That has been known now for six years! The question is what protocol will prolong life. It is not that PSA does not work; it just does not work as it was being used ten years ago. This study only shows that.

The Times further states:

"In the European study, 48 men were told they had prostate cancer and needlessly treated for it for every man whose death was prevented within a decade after having had a PSA test. Dr. Peter B. Bach, a physician and epidemiologist at Memorial Sloan-Kettering Cancer Center, says one

way to think of the data is to suppose he has a PSA test today. It leads to a biopsy that reveals he has prostate cancer, and he is treated for it. There is a one in 50 chance that, in 2019 or later, he will be spared death from a cancer that would otherwise have killed him. And there is a 49 in 50 chance that he will have been treated unnecessarily for a cancer that was never a threat to his life. Prostate cancer treatment can result in impotence and incontinence when surgery is used to destroy the prostate, and, at times, painful defecation or chronic diarrhea when the treatment is radiation."

Again that is not what the data says. The data shows that men were treated and did not die in either case. The two US cases are so overlapping that a bright line is not there and the European cases due to the longer time between screenings also merge to being identical. The statement about impotence and the like are scare statements since we know that if you have cancer and if we do not know the true level of malignancy then we just remove it, we don't want to be sued.

This leads to the final issue, genetic evaluation. Namely as we have discussed elsewhere we believe that genetic testing for predisposition, presence, staging, and prevention is slowly making progress. It is this effort which will eventually bear fruit.

In a 2005 paper in Science by Tomlins et al they state:

"A central aim in cancer research is to identify altered genes that play a causal role in cancer development. Many such genes have been identified through the analysis of recurrent chromosomal rearrangements that are characteristic of leukemias, lymphomas, and sarcomas (1). These rearrangements are of two general types. In the first, the promoter and/ or enhancer elements of one gene are aberrantly juxtaposed to a proto-oncogene, thus causing altered expression of an oncogenic protein. This type of rearrangement is exemplified by the opposition of immunoglobulin (IG) and T cell receptor (TCR) genes to MYC, leading to activation of this oncogene in B and T cell malignancies, respectively (2). In the second, the rearrangement fuses two genes, resulting in the production of a fusion protein that may have a new or altered activity..."

Their conclusion is:

"The existence of recurring gene fusions of TMPRSS2 to the oncogenic ETS family members ERG and ETV1 may have important implications for understanding prostate cancer tumorigenesis and developing novel diagnostics and targeted therapeutics. Several lines of evidence suggest that these rearrangements occur in the majority of prostate cancer samples and drive ETS family member expression. "

Thus gene expression will be essential as a diagnostic tool. In a recent 2008 NEJM article by Zheng et al they state:

"Multiple SNPs in each of the five regions were associated with prostate cancer in single SNP analysis. When the most significant SNP from each of the five regions was selected and included in a multivariate analysis, each SNP remained significant after adjustment for other SNPs and family history. Together, the five SNPs and family history were estimated to account for 46% of

the cases of prostate cancer in the Swedish men we studied. The five SNPs plus family history had a cumulative association with prostate cancer ... In men who had any five or more of these factors associated with prostate cancer, the odds ratio for prostate cancer was 9.46 ..., as compared with men without any of the factors. The cumulative effect of these variants and family history was independent of serum levels of prostate-specific antigen at diagnosis...SNPs in five chromosomal regions plus a family history of prostate cancer have a cumulative and significant association with prostate cancer."

This further indicates that significant gene progress is being made.

The key fact to take from this exercise is that the results proved something which has some merit. It did not address the true question of what PSA testing if any can reduce mortality. It proved that there was no difference between two sets of PSA testing protocols. However as we have argued one would not have expected a difference. Furthermore the work done since this trial has begun has fine-tuned this testing. The true question will ultimately be a genetic question.

The New York Times⁸ had an editorial on the prostate papers in NEJM⁹ which we commented upon yesterday. The Times says:

"The studies — one done in the United States, one in Europe — both show that screening had little or no effect in reducing prostate cancer deaths."

That is NOT what the papers said. They said that the protocols used to screen had little or no effect. NOT that "screening had little or no effect".

The question the researchers should have asked was:

"What level of PSA yields a positive result regarding the reduction of mortality?"

or even better:

"What level of PSA and what level of PSA velocity yields a positive result regarding the reduction of mortality?"

They did not ask that question. They asked the question:

"Does a PSA test of 4.0 threshold reduce mortality as compared to two sample groups."

Well, as we also said the American sample groups were both "tested" albeit not as frequently, and the European sample groups were for all purposes untested. Thus frankly the level was wrong, which was known since 2003 as in NEJM, in the paper by Punglia et al¹⁰, which showed

⁸ http://www.nytimes.com/2009/03/20/opinion/20fri3.html?_r=1&ref=opinion

⁹ <http://www.nejm.org/doi/full/10.1056/NEJMoa0810696>

¹⁰ <http://www.nejm.org/doi/full/10.1056/NEJMoa021659>

that a PSA of 2.3 was required to get reasonable levels! The 4.0 level was outdated for six years. No wonder there was no positive result, in addition to the samples used.

Consider if we did a test that said for women we screen for palpable breast lesions only larger than 4 cm in diameter. Then we would likely conclude that breast screening is ineffective since those screened and those not screened died at the same rate!

This demonstrates two issues:

First, the newspapers do not have the basic competence to read and report the facts. Words mean something and in this case lives hang in the balance.

Second, you may get answers to a question but it may very well be the wrong question. Ten years ago this may have been the right question, but we learned something. So does that mean we just continue a flawed study.

2.3.2 Summary

In this section we have provided a summary overview of PSA and its usefulness and then we have spent time looking at the many trials that have been conducted with PSA and looking at its efficacy. The problem with PSA is that it is in the midst of a massive political debate. The debate is one where with the changes in health care provisioning, namely the significant takeover of health care by the Federal Government, now accounting for almost one third of the population, and growing, the need to keep costs down drive medical care rather than providing for the patient. We have argued this extensively elsewhere.

We have addressed the following issues:

Is PSA a useful test? The answer clearly is yes but it has its problems. One would never use the PSA alone. Family history is often a more of a factor than PSA alone.

Are the Trials showing the limited use of PSA valid? We have shown that the trials, European and American, were fundamentally flawed. Although they were originally well focused, as we learned more about PSA we learned that the point at which one should perform follow up are variable and oftentimes should be done sooner at lower PSA values. The Trials used protocols that were 20 years old and new information was obtained including PSA velocity and percent free PSA.

Is PSA testing cost effective? This is the QALY issue, the cost effectiveness of the test measured in years of life saved. However given the uncertainty over indolent and aggressive cancers the determination is still at issue.

Is the PSA issue at a point of certainty that a policy can be developed and promulgated via a CER approach. The answer is clearly no. There is no consistent basis of agreement in the clinical research. Moreover there is no agreement scientifically as how best to grade PCa. After a biopsy we have at best the Gleason scoring system, albeit useful, it does not necessarily reflect the best modalities of treatment. Considerable research must still be done on the topic.

2.4 THE CONFLICT IN PSA STUDIES

In a recent Urology Today posting they discuss the variations in PSA testing and PCa, prostate cancer, in Europe and the US¹¹.

Specifically they state:

This study compared PSA screening performance for detecting CaP in the ERSPC-Rotterdam with the US population. The authors report that PSA screening performance in this analysis could provide quantitative explanations for the different mortality results of ERSPC-Rotterdam and the US Prostate, Lung, Colorectal and Ovarian trial. ..The model includes 18 detectable preclinical states in the natural history of CaP that are derived from combinations of clinical stage, grade, and metastatic stage. In this model, PSA testing and subsequent biopsy is modeled as a single test, therefore PSA test sensitivity also depends on whether a positive test is followed by a biopsy.

...The predicted CaP incidence peak in the US was higher than the observed CaP incidence Peak (13.3 vs. 8.1 cases per 1,000 man-years), suggesting a lower detection of CaP in the US than in ERSPC-Rotterdam. The lower sensitivity of PSA screening in the US compared with ERSPC-Rotterdam may be due to a higher PSA cutoff level for recommending biopsies in the US. Data suggests that the biopsy compliance rate is over twice as high in the screening arm of ERSPC-Rotterdam. However, other differences included racial differences between the US and Rotterdam, frequency of PSA testing, explanations for the drop in CaP incidence after 1992 and the inability to compute 95% confidence intervals for the sensitivity parameters.

The study found that PSA screening in the US did not detect as many CaPs as in ERSPC-Rotterdam due to the lower sensitivity of PSA testing followed by a biopsy.

This study presents in a bit convoluted way the problems with PSA testing. They are:

1. PSA tests are not consistent. One assay will give different results from another assay. The difference that we have measured can be as great as a 50% variation from assay to assay. The stated variation is less than 10% but the measured is closer to 50%. Thus a single test can have great variability.
2. Repeat testing with the same assay also has testing variances due to life style. Namely irritated prostates and the like cause variations in PSA as much as 25%.
3. PSA Velocity, VPSA, is the dominant test metric and that requires many years of tracking. It is the average of three consecutive measurements and the derivation of velocity therefrom. Thus one needs a good baseline of ten years of annual PSA data at a minimum to determine reliable PSA velocity. The three sample test is an attempt to reduce the variability from the above two causes.

¹¹

http://www.urotoday.com/61/browse_categories/prostate_cancer/editorial_prostatespecific_antigen_screening_in_the_united_states_vs_in_the_european_randomized_study_of_screening_for_prostate_cancerrotterdam03112010.html

4. There is a recent tendency to delay biopsy from an exaggerated PSA test. In fact many internists and family physicians do not pay attention to velocity because they do not have access to the data! It is questionable if they are even aware of the velocity testing.

5. The problem today is that PSA testing looks at just one PSA sample and we know they are highly variable. Thus rather than sampling bi-annually the test should be performed annually and the long term data recorded and analyzed.

The problem of having data on patient histories is pandemic. For example the PSA is but one yet so too is HbA1c, and even blood pressure as well as HDL and many other variables. Medicine is a science and art which is often driven by a change, change in some chemistry measurement, change in weight, sight, moles, and the like. Thus it is imperative that a good HIT notwithstanding that the patient develop their own records, and bring them with them to the physician. Noticing a change can save a life.

2.5 PSA AND COMPARATIVE EFFECTIVENESS RESEARCH

We have argued elsewhere against CER in the new health care bill. Our argument is that CER as so structured takes away from the open clinical field the results and codifies them in a Government panel and uses the hammer of reimbursement as the motivator for employing the new mandates. In NEJM there was a recent article describing the next steps that are to be taken with CER. They state them as follows¹²:

Institute of Medicine's Recommendations for a National System of Comparative-Effectiveness Research (CER).

- 1. Prioritization of CER topics should be a sustained and continuous process, recognizing the dynamic state of disease, interventions, and public concern.*
- 2. Public participation (including participation by consumers, patients, and caregivers) in the priority-setting process is imperative for ensuring that the process is transparent and that the public has input into the delineation of research questions.*
- 3. Consideration of CER topics requires the development of robust, consistent topic briefs providing background information, an understanding of current practice, and assessment of the research status of the condition and relevant interventions.*
- 4. Regular reporting of the activities and recommendations of the prioritizing body is necessary for evaluating the portfolio's distribution, its effect on discovery, and its translation into clinical care in order to provide a process for continuous quality improvement.*
- 5. The secretary of HHS [Health and Human Services] should establish a mechanism — such as a coordinating advisory body — with the mandate to strategize, organize, monitor, evaluate, and report on the implementation and impact of the CER program.*

¹² <http://healthpolicyandreform.nejm.org/?p=3017&query=home>

6. *The CER program should fully involve consumers, patients, and caregivers in key aspects of CER, including strategic planning, priority setting, research-proposal development, peer review, and dissemination.*

7. *The CER program should devote sufficient resources to research and innovation in CER methods, including the development of methodologic guidance for CER study design — for instance, on the appropriate use of observational data and approaches to designing more informative, practical, and efficient clinical trials.*

8. The CER program should help to develop large-scale clinical and administrative data networks to facilitate better use of data and more efficient ways of collecting new data to inform CER.

9. The CER program should develop and support the workforce for CER to ensure that the country has the capacity to carry out the CER mission.

10. The CER program should promote rapid adoption of recommendations based on CER findings and conduct research to identify the most effective strategies for disseminating new and existing CER findings to health care professionals, consumers, patients, and caregivers and for helping them to implement changes based on these results in daily clinical practice.

The analysis of these objectives leads to further insight as to where these folks are going. To reiterate, CER, as best as I understand their meaning, albeit inferentially, since one cannot find a delimited definition, it is expansively defined by what it does, a typical Government program, is a Government program targeting clinical studies, with the participation of a broad based of interested parties, who will in some undefined manner develop and recommend, perhaps mandate, clinical procedures related to the delivery of health care to Americans.

Frankly this is the antithesis of how medicine or any science is practiced. Imagine is we have had such a group in physics, chemistry, engineering, a centralized Government entity telling us what the problems are that we should consider and then seeking the input from many third party interest groups who may totally lacking in any expertise and then setting up what the truth is. Would we have an Einstein, a Schrodinger, a Feynman, a Wiener, or perhaps a Banting or Osler, where would those ideas come from that were initially non-conformists? Frankly are these people just plain Orwellian!

The authors, clear supporters of this plan, state:

First, the national CER program must develop an overall funding strategy. It could follow the traditional biomedical research model by inviting proposals on any of the 100 high-priority topics and awarding grants to the scientifically strongest proposals. However, the research interests of individual investigators would then define the national priorities. Instead, we believe that the national CER program should decide on a coordinated portfolio consisting of research on priority topics, infrastructure enhancement, and studies of translation and adoption.

Medical research has been around for over a century and it continues to evolve as we learn more. It is iterative and it modifies itself as we learn more. Some studies are well posed at their initiation but flawed by the time they are completed. I come back to the classic prostate cancer studies. They were started when a PSA of 4.0 was considered the gold standard. Over the years we have found that a PSA of 2.0 is as important for a younger man as 4.0 is for an older and also that PSA velocity is more a predictor. It is iterative and in some ways combative. A national CER program is consensus driven, worst of all worlds.

Second, the *CER* program should establish an initial list of priority topics and evaluate the current state of knowledge about each. For the first of these tasks, it should build on the priority-setting work of the *IOM* committee. It could develop a portfolio chosen from the top 25 *IOM* topics by applying the already-published prioritization criteria of the *IOM*

The portfolio is already there as a matter of ongoing research. Why redo the effort? Is this nothing more than justification for billions of more dollars spent by the Government. The money is spent well now why do we need change.

Third, the *CER* program, with the help of expert advisory committees and the research community, should choose the research methods that will fill gaps in the evidence for a specific topic. In an investigator-initiated research program, the grant applicant typically chooses the methods. The cost of studies using the methods of *CER* (whether clinical trial, observational study, or qualitative research) varies widely.

Evidence is always changing. Back to the prostate example. We know also that 5-10% of prostate cancers are highly aggressive. The question is why? Perhaps the four or five gene hits, ultimately knocking out PTEN, leads to the aggressiveness. Perhaps many men have genetically had the hits and they are predisposed, possibly there are epigenetic factors as well. These are the issues we should be working on, and these are the issues which the highly motivated and competent researchers are already working on. Why do we need another group? That question has never been answered. Perhaps to create approved methods to just "kill of the old folks" and replace the "death panels" with "death procedures".

Fourth, the program should strive for a balanced portfolio of high-impact research topics. Although it could simply rank topics in order of importance and fund them in ranked order until the money ran out, we recommend developing a portfolio that addresses a balanced distribution of topics, outcomes, and target populations, as well as keeping the total portfolio cost within budget and producing a body of evidence sufficient to influence health care decisions.

The nature of the portfolio changes as we learn more each step. Dynamic portfolios are common in the way we do research now. The "hot topic" appears and researchers follow the path. Having a bunch of Government chart preparers do this is frankly insane!

Fifth, the CER program should evaluate progress and report to the public. To meet this obligation, it should do large-scale, ongoing observational research and evaluation to measure CER's effects on clinical practices and patient outcomes.

This I really do not understand. Medical research is always publicly available, NEJM is on line, as is JAMA and the list continues. Clinical trials are an everyday affair, just read NEJM and JAMA and the hundreds of other journals. So what is the point? Just spending more money.

The only possible reason for CER is Government control. Control over what the Government will pay for and worse the control over what physicians can do. This is not the code of civil procedure used in Federal Courts, this is science, and as such changes. Having the Government as the regulator of change is not just stupid it is immoral.

The British Journal of Cancer has just published an interesting article regarding Prostate Cancer¹³. They state:

There is evidence that prostate cancer (PC) screening with prostate-specific antigen (*PSA*) serum test decreases PC mortality, but screening has adverse effects, such as a high false-positive (*FP*) rate. We investigated the proportion of *FPS* in a population-based randomized screening trial in Finland...An *FP* result is a common adverse effect of PC screening and affects at least every eighth man screened repeatedly, even when using a relatively high cutoff level. False-positive men constitute a special group that receives unnecessary interventions but may harbor missed cancers. New strategies are needed for risk stratification in PC screening to minimize the proportion of *FP* men.

The last statement is the most powerful. It states that despite the false positive, namely a man is told that an increased *PSA* may be an indicator for Prostate Cancer, and then after a biopsy there does not appear to be any, then shortly thereafter they do come down with PCa. Namely false positives may not truly be false positives but early true positives. Specifically the histological test of looking at cells may not be the correct early assessment method.

The Cancer Research UK states in their assessment of the article the following¹⁴:

The study, a clinical trial of the controversial *PSA* test for prostate cancer, tells us that false-positives are common. It also shows that men who get a false alarm:

- 1) are likely to get another one the next time they go for a *PSA* test
- 2) are likely to refuse future invitations to screening, and
- 3) are likely to actually be diagnosed with prostate cancer the next time round

The third result, in particular, is a fascinating one. It suggests that men who get a false-positive result through *PSA* testing, in the words of the researchers, “constitute a special group”. They could well go through unwarranted tests, but they could also harbor missed cancers that only turn up later.... As we mentioned above, there’s a large prostate screening trial running across Europe,

¹³ <http://www.nature.com/bjc/journal/v102/n3/abs/6605512a.html>

¹⁴ <http://scienceblog.cancerresearchuk.org/2010/01/20/the-meaning-of-false-alarms-in-prostate-screening/>

called *ESPRC*. The new results, published in the British Journal of Cancer, (which Cancer Research UK owns) come from the Finnish part of this trial – its largest component. It involves more than 80,000 men, some of whom were randomly invited to three rounds of *PSA* testing, with four-year gaps between each round. Roughly 30,000 men attended their first round of screening and more than 10,000 of these men went on to attend all three rounds.

The study showed that false-positives are a common part of *PSA* testing. In any individual round of testing, the majority of positive results are false alarms (between 60 and 70 per cent), while just over a quarter lead to an actual cancer diagnosis. Among the men who attended at least one round of screening, 1 in 8 had at least one false-positive result.

It's worth noting that the researchers were using a fairly high cut-off level of *PSA* (4 ng/ml) – i.e. the level above which they were thought to have suspected prostate cancer. This sets a pretty high bar for a positive result and should minimize the number of false positives. Nonetheless, many still crept through.

Among the men who get a false alarm in one round, more than half will get another false alarm in the next one. Many men without tumors have persistently high *PSA* levels for some other reason, so they keep on testing positive. That's a lot of extra worry and more potential for unneeded tests.

Indeed, in this trial, every third man who got a false alarm went through two biopsies within 4 years of their result. That's probably an underestimate too, as it *doesn't* account for any visits to private doctors.

However, the study also shows that false-positives *aren't* entirely meaningless. If men had a false alarm during one round of screening, they were 3-9 times more likely to be diagnosed with prostate cancer during the next round...."

The analysis of the poor trials mentioned above is what we had commented on a year ago when the results were issued. Namely they used the 4.0 *PSA* level which we now know to be wrong, especially for men under 65. In addition we also now know that the better measure is *PSA* velocity, namely the change in *PSA* in a year's time. If the change is 0.75 or greater then there is a 90% chance of Prostate Cancer. That is a fairly good metric. Thus if you have a *PSA* of 1.5 in one year and the next year it is 2.25, you have a 90% chance of incipient PC.

3 BASIC PROSTATE CANCER TESTS

The 2003 NEJM article by Nelson et al on Prostate Cancer lays out the genetic progression of Prostate Cancer and it is that progression which PSA somewhat follows. Yet it is that progression that most histological exams, using say a Gleason framework, do not follow. It is worth a simple review to see what we mean. Cancer is simply a breaking down of the normal cell cycle. Cells duplicate themselves via mitosis and it is that mitotic process wherein old cells "die" and new cells are created. In fact the old cell just repairs itself and then duplicates itself.

We will now examine some of the issues surrounding prostate biopsies as well and some quantitative procedure relating to PSA.

3.1 PROSTATE BIOPSY SAMPLING

The question of detecting prostate cancer upon biopsy is an interesting exercise in sampling. We proceed to develop a simple model to determine the probability of missing cancer upon a biopsy. To accomplish this we have to make a set of basic assumptions. These may be modified and they in turn will modify the results.

We first look at a simple model of cancer and focus on the prostate. The issue is simply at what point should we be concerned. How large a collection of cells is a collection to be concerned about. Thus we first review some issues of growth and size and then we examine the issue of sampling and detection probability.

3.1.1 Some Preliminary Facts

Cancer is a complicated disease and even at that it is an understatement. The control mechanisms which set cancer in motion flow through the many pathways which are known. Yet there is at the gross level some simplicity which we want to develop here for a clinical purpose of evaluating the effectiveness of prostate biopsies. There are a few basic facts.

1. Cancer is generally clonal, one cell goes wild and keeps reproducing. This is uncontrolled mitosis or cell replication. Thus mitosis and its control is of major concern. The clonal theory states that it is but one cell that goes into an uncontrolled state and that all progeny are progeny from that parent cell. There is some work recently with regard to stem cells which counters this theory but for our present interests we shall keep the clonal approach. We shall examine the stem cell theory later in the text.

2. The reproduction rate is not quite doubling, some progeny do not survive, thus depending on the status of the tumor the growth rate is between 1 and 2 per generation. Sometimes it is less than 1 and it even regresses as seen in melanoma. The ability of clonal progeny to survive is also a window to cancer control.

3. There are cell cluster sizes which are of interest. As Weinberg notes, when there are 10^6 cells the tumor can be seen under CAT or MRI. When there are 10^9 cells it is palpable, when there are 10^{12} the patient dies. Almost always these metrics can be used. Thus when we look at prostate cancer we are looking for the needle in the haystack, hopefully, namely the 1 million cell clusters.

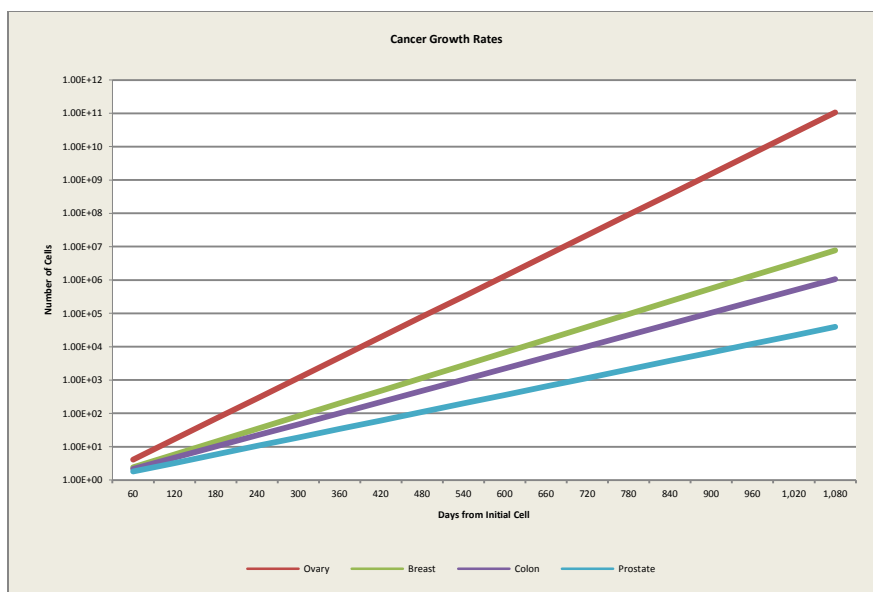
4. Cancer growth and evolution is a classic epigenetic systems process in cell growth and replication. The cell loses its ability to die, it just keeps growing and replicating itself with its functionality reduced to it replication and nothing else.

5. The mitotic cycle, the time from quasi doubling to quasi doubling is different for many cancers. Ovarian cancer has a short doubling time, days. The mitotic cycle itself is about 16 hours and then the initiation of another cycle may start within a few days. This is why ovarian cancer is so aggressive. In contrast indolent prostate cancer may take months between doubling and the reproduction rate itself may be quite low, well less than 2 and slightly more than 1. However, there is an aggressive form of prostate cancer, the details of which are still not well known, where the rate and the reproduction rate make for rapid growth. The rate may be quite short, days or weeks for doubling and the reproduction rate may be near 2.

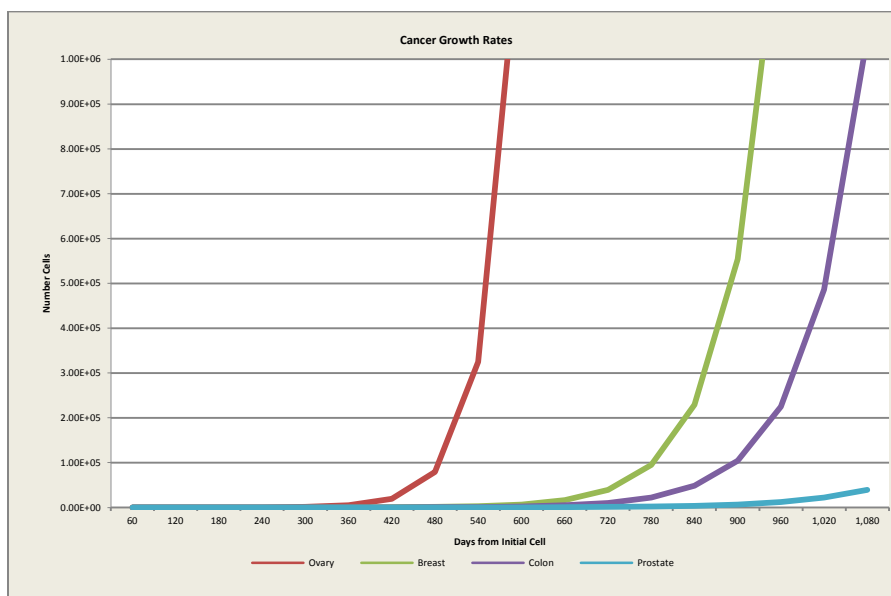
This is the simple story of cancer so that we can look at the 1 million cell cluster. We show a simple growth model for 4 cancers below. We depict the number of cells from the clonal beginning for each of these cancers as a function of time. One can see how ovarian and breast cancers kill so quickly.

| <i>Time (Days)</i> | <i>Ovary</i> | <i>Breast</i> | <i>Colon</i> | <i>Prostate</i> |
|--------------------|--------------|---------------|--------------|-----------------|
| 60 | 4.10E+00 | 2.41E+00 | 2.16E+00 | 1.80E+00 |
| 120 | 1.68E+01 | 5.83E+00 | 4.67E+00 | 3.24E+00 |
| 180 | 6.87E+01 | 1.41E+01 | 1.01E+01 | 5.83E+00 |
| 240 | 2.81E+02 | 3.40E+01 | 2.18E+01 | 1.05E+01 |
| 300 | 1.15E+03 | 8.21E+01 | 4.70E+01 | 1.89E+01 |
| 360 | 4.72E+03 | 1.98E+02 | 1.02E+02 | 3.40E+01 |
| 420 | 1.93E+04 | 4.79E+02 | 2.20E+02 | 6.12E+01 |
| 480 | 7.92E+04 | 1.16E+03 | 4.74E+02 | 1.10E+02 |
| 540 | 3.25E+05 | 2.79E+03 | 1.02E+03 | 1.98E+02 |
| 600 | 1.33E+06 | 6.75E+03 | 2.21E+03 | 3.57E+02 |
| 660 | 5.44E+06 | 1.63E+04 | 4.78E+03 | 6.43E+02 |
| 720 | 2.23E+07 | 3.93E+04 | 1.03E+04 | 1.16E+03 |
| 780 | 9.13E+07 | 9.50E+04 | 2.23E+04 | 2.08E+03 |
| 840 | 3.74E+08 | 2.29E+05 | 4.82E+04 | 3.75E+03 |
| 900 | 1.53E+09 | 5.54E+05 | 1.04E+05 | 6.75E+03 |
| 960 | 6.28E+09 | 1.34E+06 | 2.25E+05 | 1.21E+04 |
| 1,020 | 2.57E+10 | 3.23E+06 | 4.86E+05 | 2.19E+04 |
| 1,080 | 1.05E+11 | 7.80E+06 | 1.05E+06 | 3.93E+04 |
| 1,140 | 4.31E+11 | 1.88E+07 | 2.27E+06 | 7.08E+04 |
| 1,200 | 1.77E+12 | 4.55E+07 | 4.90E+06 | 1.27E+05 |
| 1,260 | 7.24E+12 | 1.10E+08 | 1.06E+07 | 2.29E+05 |
| 1,320 | 2.96E+13 | 2.65E+08 | 2.29E+07 | 4.13E+05 |
| 1,380 | 1.21E+14 | 6.41E+08 | 4.94E+07 | 7.43E+05 |
| 1,440 | 4.97E+14 | 1.55E+09 | 1.07E+08 | 1.34E+06 |
| 1,500 | 2.04E+15 | 3.74E+09 | 2.30E+08 | 2.41E+06 |
| 1,560 | 8.34E+15 | 9.03E+09 | 4.98E+08 | 4.34E+06 |
| 1,620 | 3.42E+16 | 2.18E+10 | 1.08E+09 | 7.80E+06 |
| 1,680 | 1.40E+17 | 5.27E+10 | 2.32E+09 | 1.40E+07 |
| 1,740 | 5.73E+17 | 1.27E+11 | 5.02E+09 | 2.53E+07 |
| 1,800 | 2.35E+18 | 3.07E+11 | 1.08E+10 | 4.55E+07 |
| 1,860 | 9.62E+18 | 7.42E+11 | 2.34E+10 | 8.19E+07 |
| 1,920 | 3.94E+19 | 1.79E+12 | 5.06E+10 | 1.47E+08 |
| 1,980 | 1.61E+20 | 4.33E+12 | 1.09E+11 | 2.65E+08 |
| 2,040 | 6.61E+20 | 1.04E+13 | 2.36E+11 | 4.78E+08 |
| 2,100 | 2.71E+21 | 2.52E+13 | 5.10E+11 | 8.60E+08 |
| 2,160 | 1.11E+22 | 6.09E+13 | 1.10E+12 | 1.55E+09 |
| 2,220 | 4.54E+22 | 1.47E+14 | 2.38E+12 | 2.79E+09 |

Note that in this model we assume rapid growth for ovarian cancer and slow growth for prostate. This may not always be the case. For example there are certain prostate cancers which grow very aggressively, the reasons are not yet known. We show the growth below in two scales.



The above is on logarithmic scale and the one below is linear.



This shows two factors. One is that certain cancers grow so quickly that one must have to screen on a quarterly basis to have any effect. That is very costly. Second some cancers grow so slowly that screening will result in surgeries that are not necessary since the cancer will never grow large enough to kill the person. Thus between too fast and too slow are many others, and too fast may not be too fast and too slow may not be too slow. That is the conundrum.

In a recent JAMA article the author's state¹⁵:

¹⁵ <http://jama.ama-assn.org/cgi/content/full/302/15/1685>

"Early detection may not be the solution for aggressive cancers because many may not be detected early enough for cure. Some small "curable" breast cancers, categorized as low risk by National Institutes of Health criteria, have a high mortality risk when analyzed using prognostic molecular profiles such as the NKI 70 gene test. Biologically aggressive cancers present with a higher stage despite screening. Interval cancers, those that present clinically between routine screens, have a higher growth fraction and are more likely to be lethal compared with screen detected cancers.

In the neoadjuvant I-SPY (Investigation of Serial Studies to Predict Your Therapeutic Response With Imaging and Molecular Analysis) trial, in which the mean tumor size was 6 cm (accrual 2003-2006 in the United States), 91% had poor prognosis biology²⁷ (using the NKI 70 gene test), which is much higher than the 33% poor prognosis proportion in women undergoing routine screening.

Of women undergoing routine screening in the I-SPY TRIAL, 85% of the malignancies were interval cancers and only 15% were screen detected, suggesting that locally advanced cancers reflect the growth curve.... Similarly, the most lethal prostate cancers are those with rapidly increasing...

Screening is most successful when premalignant lesions can be detected and eliminated as in the case of adenomatous polyp removal during colonoscopy screening or cervical intraepithelial neoplasia ablation by colposcopy after detection by pap smear. Perhaps most important is that screening for cervical and colon cancer and the removal of pre-neoplastic lesions have been accompanied by a significant decrease in their invasive cancer counterparts; this has not been seen in breast and prostate cancer.

Ductal carcinoma in situ, rare prior to widespread screening, now represents 25% to 30% of all breast cancer diagnoses (>60 000 new case-diagnoses annually are not included in the invasive cancer statistics), the majority of these lesions are low and intermediate grade. Ductal carcinoma in situ is considered to be a precancerous lesion and standard of care is excision and adjuvant treatment. However, after 2 decades of detecting and treating DCIS, there is no convincing evidence of substantial reduction in invasive breast cancer incidence. The 2002 decrease in incidence leveled off in 2005 and is attributed to a reduction in postmenopausal hormone therapy use, not DCIS removal."

The authors then suggest actions which we have detailed earlier¹⁶, they rephrase them as follows:

Biomarkers to Differentiate Significant- and Minimal-Risk Cancers. To help move toward a more effective solution, the first step is a change in mindset in scientific discovery efforts and clinical practice....

Reduce Treatment Burden for Minimal-Risk Disease. Many diagnosed tumors will follow an indolent course for the patient's lifetime⁴² or are probably cured with surgical excision alone.

¹⁶ See McGarty, Health Care Policy, 2009.

Develop Tools to Support Informed Decisions. Information about risks of screening and biopsy should be shared with patients before screening. At the time of cancer detection, risks and benefits of treatment for specific biological subtypes should be shared

Focus on Prevention for the Highest-Risk Patients. Ultimately, prevention is preferable to screening by reducing the risk that a patient will have a diagnosis, experience undesirable effects of treatment, and confront the specter of recurrence. For both breast and prostate cancer, available agents are proven to reduce cancer risk: finasteride and tamoxifen or raloxifene.

Demonstration Projects: Tactics for the New Strategy. To reduce morbidity and mortality from breast cancer and prostate cancer and to execute the proposed strategy, a comprehensive approach, using large demonstration projects to create a learning system, integrating both clinical care and research is needed. By spanning the spectrum from screening to treatment and survivorship, learning from diagnosis, treatment, and outcomes can be applied to developing tailored strategies for screening and prevention."

The problem is a bit more complex, however. It requires screening first, then staging. Screening is a difficult one since what is known today about the genetics of cancer growth for the most part reflects what is activated in a rapidly growing cancer. There are certain genetic predisposing genes but the problem is what turns them on and when.

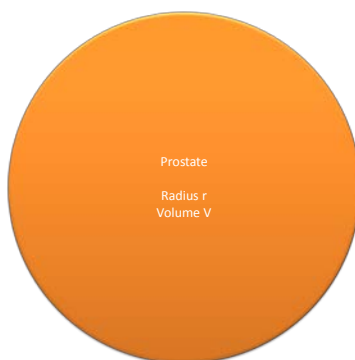
3.1.2 Basic Assumptions

We begin by examining a theoretical “prostate” which we assume is spherical and then examine the effectiveness of sampling. We now make certain assumptions.

Assume a spherical prostate. This is not unrealistic and we then state that the prostate volume is V and it has an effective radius of r . Recall that:

$$V = \frac{4}{3}\pi r^3$$

We depict such a model below.



Now assume that there are cells in the prostate and that a cell is of a radius r_{cell} and that the number of cells in the prostate is determined as:

$$N_{cells} = \frac{V_{prostate}}{V_{cell}} = \frac{r_{prostate}^3}{r_{cell}^3}$$

We present some of the basic assumptions below. We assume a standard prostate of 40 cc and a standard cell size of 100 μm and from this we can readily obtain number of cells of almost 10 million in a prostate. There may be fewer due to packing ratios and glands but for the purpose of the analysis this is not unreasonable.

When performing a biopsy we use a needle of 1.2 mm in diameter and of length 15 mm for each core sample¹⁷.

| | Basic Units | Units μm |
|----------------------------|-------------|---------------------|
| Prostate Size (cc) | 40.00 | 40,000,000,000,000 |
| Prostate Radius | 2.12 | 21,219 |
| Cell Size μm | 100.00 | |
| Cell Volume | | 4,186,667 |
| Cells per Prostate | | 9,554,140 |
| Probe Diameter mm | 1.20 | 12 |
| Probe Length mm | 15.00 | 15.00 |
| Core volume cmm | | 1,696 |
| Core Volume cum | | 1,695,600,000,000 |
| Core Vol % Prostate Volume | | 4.239% |
| Cells in Core | | 405,000 |
| Number Cores | 14.00 | |
| Total Cells in All Cores | | 5,670,000 |
| Percent Cells Sampled | | 59.346% |
| | | |
| Tumor Size in Cells | | 1,000,000 |
| Tumors Size as % Prostate | | 10.47% |

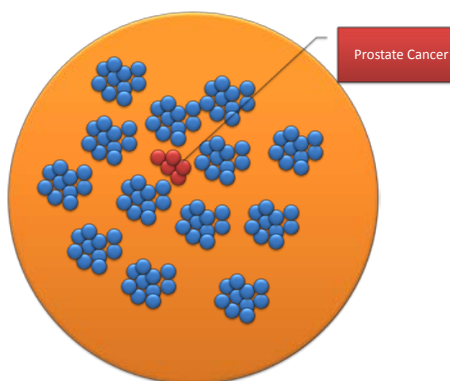
Assume that the tumor cells are of the same size as normal cells and that the tumor volume has reached a minimal perceptible size of 1 million cells.

We now set the level of cells as 1 million or 10% of the prostate cell size for a 40 cc prostate. We can alter this based upon a set level. This means that 10% of a normal prostate is composed of cancer cells. Based upon our analysis done above, this may or may not be an important issue. It is the result of 20 binary cell divisions. There may have been thirty cell divisions to get to that mass. The time between divisions may be weeks or months depending on the loss of control in

¹⁷ <http://www.jurology.com/article/S0022-5347%2807%2900738-0/abstract>

the cell. If we assume a month between division then we have a three year window from when the first malignant cell was created and when a sample of 1 million are present.

We graphically depict this situation below. We show a cluster of normal cells and a single malignant cluster. It should be noted that there may be diffuse malignant clusters and not just one depending on the growth.



3.1.3 Analysis of the Detection

We now proceed to determine the detection of the malignant cells as well as the probability of not detecting them. The literature states:

The rectal wall is thin, so it is possible to place the needle more accurately and with less injury to other tissues. When activated, the needle can remove a slender cylinder of tissue (about 1/2" by 1/16"), called a core, in a fraction of a second. Biopsy needles are tiny -- only 1.2 millimeters in diameter and less than 1/2" long -- and very precise. A sliding sheath opens once the needle enters the prostate, closes onto a sample of tissue and the needle is withdrawn¹⁸.

And

It is widely reported that a prostate biopsy gun needle advances 0.5 cm and then obtains the subsequent 1.5 cm of tissue. Based on this presumed skip area it is recommended that the needle tip must be placed 0.5 cm from the capsule before firing to obtain the capsule with the specimen. Contrary to this longstanding recommendation, in our experience we have observed that there is no such skip area. We determined the actual content of a needle core by obtaining biopsies from an apple model with clinical correlation to validate our findings¹⁹.

We make the following assumptions.

¹⁸ <http://www.upmccancercenters.com/cancer/prostate/biopsyneedle.html>

¹⁹ <http://www.jurology.com/article/S0022-5347%2807%2900738-0/abstract>

1. Assume that we use a core which has a diameter of some know amount and a core length of some known amount. This yields the volume per core.
2. Assume that the prostate has a known volume and that the cell has a known radius. Assume that the cells have a volume based upon their spherical radius and that the number of cells is simply the ratio of the prostate spherical volume to the volume of a single cell. We know that the number of cells may be a fraction lower due to packing and due to a mixture of cells in the stroma. However we can always adjust for that change.
3. Assume that we use several cores and that the location of the cores are independent and non-overlapping.
4. Assume total randomness in the cancer location. Assume that there are 1 million cancer cells as we have suggested above.

Then we want to find the probability that we can detect the 1 million cancer cells using the above set of assumptions.

Let

$$V_{Cell} = \frac{4}{3} \pi r_{Cell}^3$$

$$V_{Prostate} = \frac{4}{3} \pi r_{Prostate}^3$$

$$N_{Cells} = \frac{V_{Prostate}}{V_{Cell}}$$

$$Cell\ density = \rho_{Cell} = \frac{N_{Cell}}{V_{Prostate}} (cells / cc)$$

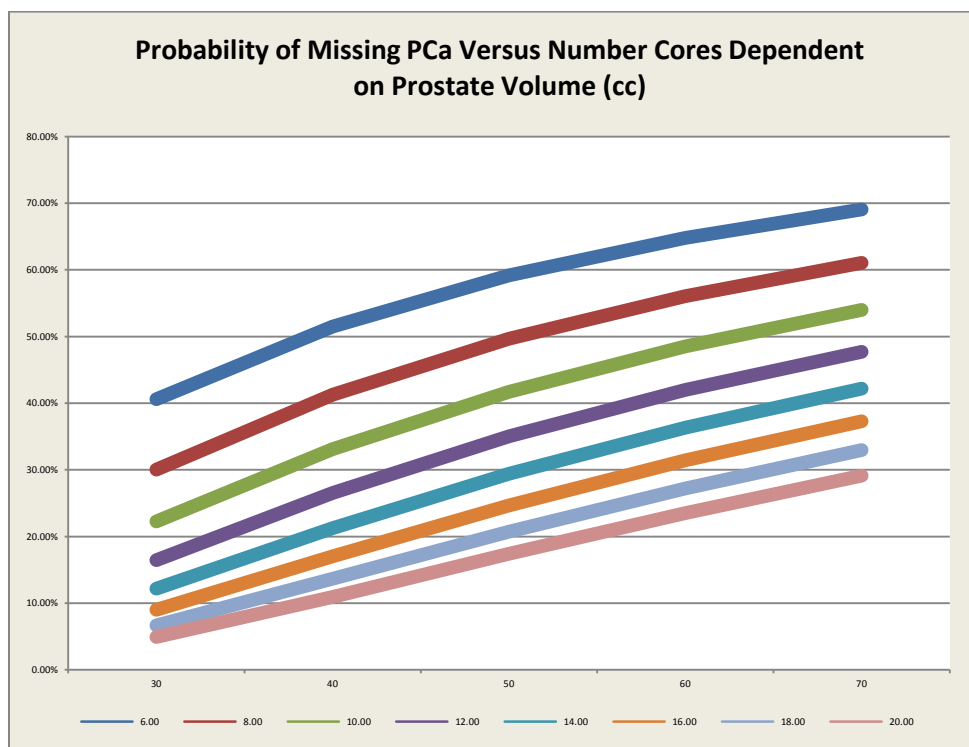
Now we assume that the cluster of cancer cells is uniformly distributed across the prostate so that the probability of placing a core in a cancer cell is determined by the chance of hitting the cancer cells with a probe. We determine this as follows:

$$p = P[Hitting\ a\ Cancer\ Cluster\ with\ one\ core] = \frac{N_{CancerCells}}{N_{ProstateCells}}$$

$$P[Miss\ a\ cluster\ with\ single\ core] = 1 - p$$

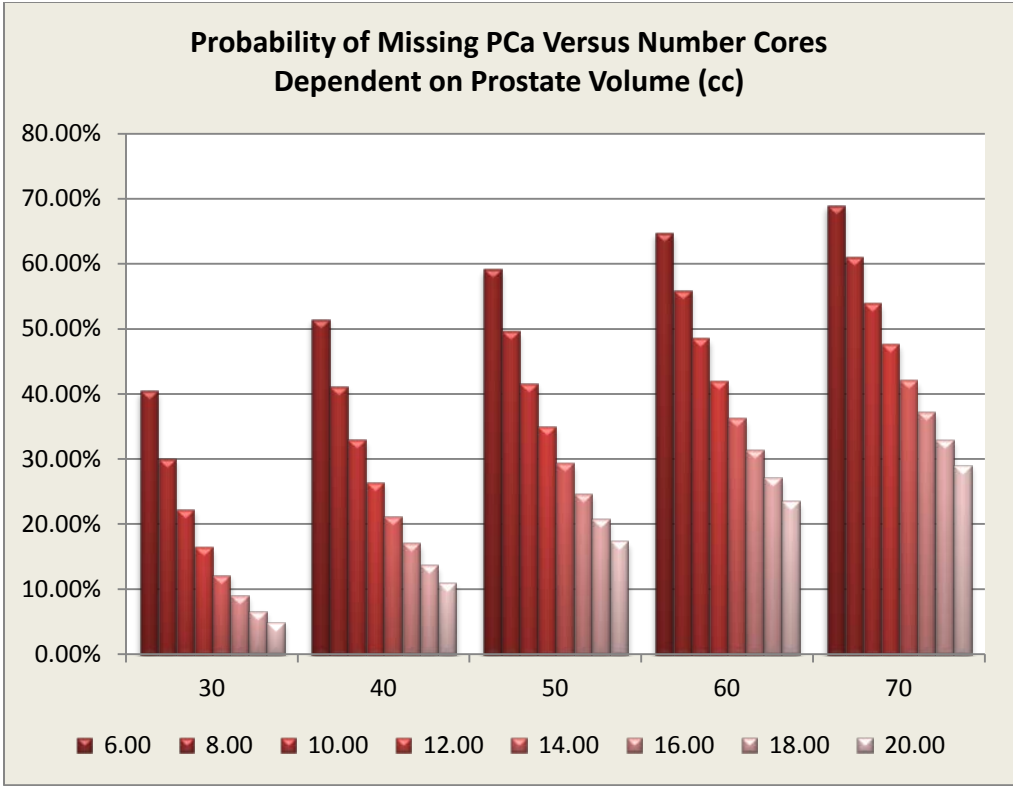
$$P[Miss\ Cluster\ with\ N\ Cores] = (1 - p)^N$$

Then using the data provided above for the samples we obtain the following curves for the miss probability.



The high miss rate even for average prostates for 10-12 cores is significant. This will play a role in later studies which often neglect this dominant factor. The only gold standard is the biopsy of the total prostate. This is why when a prostatectomy is performed the Gleason grade is often increased. The samples are just too small.

We also show below the same data as above but we present it in a different manner. Here we show by different prostate volumes the required number of cores to reach a certain level of cancer miss.



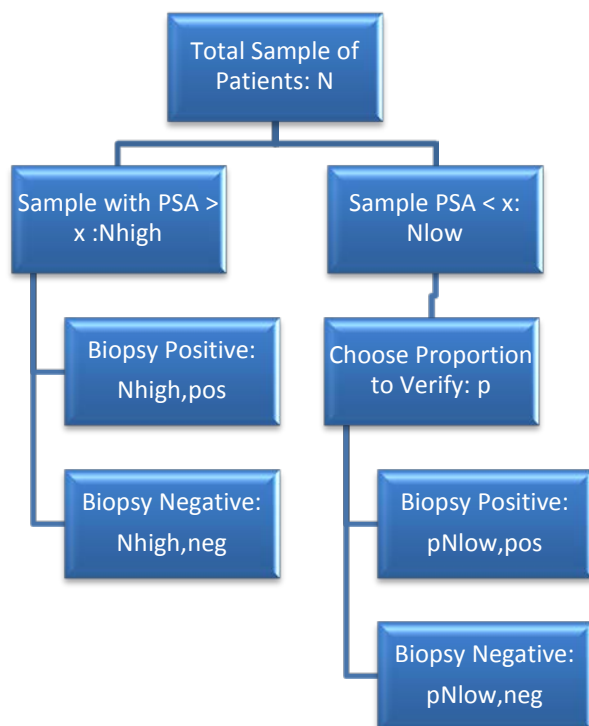
Thus we will argue that the prostate biopsy, albeit useful, is not a gold standard. It has not generally biopsied the tumor space even if assisted with ultrasound.

3.2 VERIFICATION BIAS

There are many biases in statistical tests and the verification bias is but one, but a critical one in medical testing. Let us consider a test whose usefulness we wish to test in determining the presence of a disease. In this case we will assume that we use a PSA test and we are looking for prostate cancer. We may use the test to screen and if the PSA is above a certain level we will then perform a biopsy.

We would assume the biopsy is the gold standard but as we have just shown it is clearly not due to its own sampling errors. With those whose test is below a threshold we should do a biopsy but that is costly and invasive so we choose a small sample only. This may most likely lead to a test with a verification bias.

The procedure may look as follows where we take a total of N patients, then test them and divide them into those with excess PSA and those with less than excess. Now since we assumed that excess has PCa we biopsy all of them and thus find the number with PCa and those without. Remember, however, that just because we obtain a negative biopsy there is still a chance we have a PCa. Now for the group with low PSA, there may be quite a few there, so we biopsy a small proportion, finding some with PCa and others are clear. Again note that we have always the chance that the biopsy is in error for those with the benign result.



We now want to examine the potential deficiencies with such a process.

3.2.1 The Problem

The problem is that although we choose all high values to test and validate we choose only a portion of the low values to test.

We seek a Table of the following form:

| Test Result | Disease State | |
|----------------|---------------|--------|
| | Present | Absent |
| | Positive | N(P,P) |
| | Negative | N(P,N) |

Now the above assumes we use all patients in a large sample. What if we use all who are positive but the fraction which are negative for PCa. We get the following table:

| Test Result | Disease State | |
|----------------|---------------|---------|
| | Present | Absent |
| | Positive | N(P,P) |
| | Negative | Np(P,N) |

Where Np represents the proportion of the negative test sampled. It should be noted that if we choose p as the fraction sampled that we are not in any way assured that $Np=p*N$. That is the fraction who are diseased in the subset may not equal the pro-rated fraction diseased from the larger set.

Now we wish to determine the following:

$$\text{Number}[D = PCa | PSA > n] = n_{PCa+, PSA+}$$

$$\text{Number}[D = PCa | PSA < n] = n_{PCa+, PSA-}$$

$$\text{Number}[D = noPCa | PSA > n] = n_{PCa-, PSA+}$$

$$\text{Number}[D = noPCa | PSA < n] = n_{PCa-, PSA-}$$

and

$$\text{Sensitivity} = \frac{n_{PCa+, PSA+}}{n_{PCa+, PSA+} + n_{PCa+, PSA-}}$$

and

$$\text{Specificity} = \frac{n_{PCa-, PSA-}}{n_{PCa-, PSA-} + n_{PCa-, PSA+}}$$

However this is a measure based upon samples and not a measure based upon probabilities. Let us consider a simple example of trying to detect two signals in noise. Let us assume we have:

$$s_0(t) + w(t)$$

and

$$s_1(t) + w(t)$$

and

$$r = \int_0^T r(t) dt$$

and

$$r_1 = s_1 + w$$

and

$$r_0 = s_0 + w$$

We will assume that when we do this we have the following for the variables, and we assume w is Gaussian with mean 0 and standard deviation σ . We then define the detection probability and the false alarm probability as follows:

$$P[D] = P[s_1 | s_1] = P[\text{Say } s_1 | \text{Was } s_1]$$

$$P[FA] = P[s_1 | s_0]$$

These are the detection and false alarm probabilities. The detection probability is also the sensitivity. Now:

$$\text{Assume } s_0 = 0$$

$$\text{Assume } s_1 = E$$

Then :

$$p(r / s_1) = \frac{1}{\sqrt{2\pi}\sigma} \exp\left(-\frac{1}{2\sigma^2}(r - E)^2\right)$$

and

$$p(r / s_0) = \frac{1}{\sqrt{2\pi}\sigma} \exp\left(-\frac{1}{2\sigma^2}(r)^2\right)$$

Then we can plot $P[D]$ versus $P[FA]$ and this is the ROC or receiver operating characteristic. We do this calculation by varying the selection boundary of choosing what was sent. Analytically we have:

$$P[D] = \int_T^\infty p(r / s_1) dr$$

$$P[FA] = \int_T^\infty p(r / s_0) dr$$

Clearly if we make T small then we get a better P[D] but we get a larger P[FA] as well. It of course also depends on E and the variance of the noise.

Now returning to the counting case, we can determine the probabilities from the data. Simply:

$$P[D] = \int_T p(r/s_1) dr$$

as

$$\hat{P}[D] = \frac{\text{Number of Cases PCa when PSA} > x}{\text{Total Number of PCa for all}}$$

but

$$P[D] = \lim_{n \rightarrow \infty} \hat{P}[D; n]$$

That is the measure variable approaches the true statistic only as the sample gets very large. Thus even if we were to sample all in all categories we would have some error due to limited sampling.

We now have a different problem. Let us return to the analysis we presented at the commencement of the section. Here we have a set of N patients upon whom we perform a diagnostic test, the PSA, and then we break it into two groups, those above and below a threshold, and then we biopsy all those above and only a select number below. What can we say about this test. The term validation bias has been used to determine if we have created some distortion on the end result. Frankly we totally disregard a set of the tested but un-biopsied group then clearly we have created a bias. If so how do we modify that?

Recall:

$$\hat{P}[D] = \frac{\text{Number PSA} > x \text{ and Biopsy Positive}}{\text{Number PSA} > x \text{ and Biopsy Positive} + \text{Number PSA} < x \text{ and Biopsy Positive}}$$

But we have delimited part of the above denominator by selecting out a limited number as follows:

$$\hat{P}[D] = \frac{\text{Number PSA} > x \text{ and Biopsy Positive}}{\text{Number PSA} > x \text{ and Biopsy Positive} + \text{Number PSA} < x \text{ and Biopsy Positive and in Group } V^+}$$

Clearly we will then overestimate P[D] because there may be fewer in the group. There is also the problem that we may not have detected cancers because as we have shown before, a negative biopsy does not mean cancer. We should be saying a negative biopsy and not PCa! We will return to that later.

3.2.2 Approaches to Eliminating Verification Bias

There are several ways to address the bias. We examine two of them here. The first is that of Punglia. In that paper they take data and then adjust the cells that have not been verified in a manner using the other variable they have at hand. The second approach uses a maximum likelihood approach by Jhou.

1.1.1.1 Punglia Approach

The Punglia model is shown below. We have presented it as they have. Note the large number not tested.

| Test | Disease | |
|------------|---------|--------|
| | Present | Absent |
| Positive | 92 | 27 |
| Negative | 46 | 72 |
| Not Tested | 89 | 108 |

Now recall:

$$\text{Sensitivity} = \frac{\text{Number}(\text{TestPositive}; \text{Disease Present})}{\text{Number}(\text{Disease Present})}$$

$$\text{Specificity} = \frac{\text{Number}(\text{DiseaseAbsent}; \text{TestNegative})}{\text{Number}(\text{DiseaseAbsent})}$$

For this case:

$$\text{Sensitivity} = 92 / (92 + 46) = 67\%$$

$$\text{Specificity} = 72 / (72 + 27) = 71\%$$

Now they adjust the negatives, which are all the not tested as follows. They take the patients not sampled, all of whom have a negative test, and then adjust the entries to reflect the occurrence of PCa in such a group.

We can detail the Punglia data as follows, which makes it align with the Jhou formulation.

| Disease | Test | | Total |
|------------|----------|----------|-------|
| | Positive | Negative | |
| Present | 92 | 46 | 138 |
| Absent | 27 | 72 | 99 |
| Not Tested | 0 | 197 | 197 |
| Total | 119 | 315 | 434 |

Note that we here align the 197 total negative tests in the not tested category. There were 434 total patients. Now the question is how to assign the 197 non-tested to PCa and to non PCa status. The authors make the jump apparently by using a logistic analysis based upon several variables; namely DRE, race, family history, and category of PSA, as well as aged (under and over 60). Thus they created a logistic model where:

$$\ln \frac{P[PCa]}{1 - P[PCa]} = \alpha + \sum_{i=1}^N \beta_i x_i$$

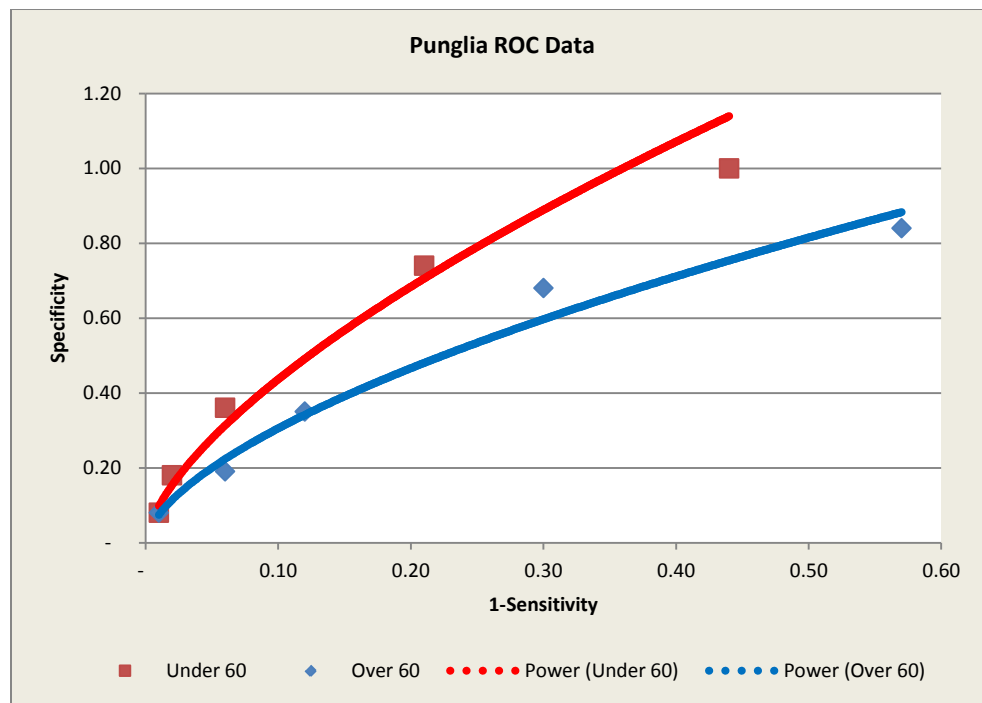
They then did a regression analysis on some data set to determine the logistic constants and adjusted the table accordingly. The result is below:

| Disease | Test | | |
|---------|----------|----------|-------|
| | Positive | Negative | Total |
| Present | 92 | 115 | 207 |
| Absent | 27 | 180 | 207 |
| Total | 119 | 295 | 414 |

This yields a Specificity of 87% and a Sensitivity of 44%. The problem is that placing so many with negative biopsies in the Disease state is done under the logistic analysis and is questionable.

| Under 60 | | | Over 60 | | |
|----------|-------------|-------------|---------|-------------|-------------|
| PSA | Sensitivity | Specificity | PSA | Sensitivity | Specificity |
| 0.90 | 100% | 56% | 1.10 | 84% | 43% |
| 1.40 | 74% | 79% | 2.10 | 68% | 70% |
| 2.60 | 36% | 94% | 4.10 | 35% | 88% |
| 4.10 | 18% | 98% | 6.10 | 19% | 94% |
| 6.10 | 8% | 99% | 10.10 | 8% | 99% |

What Punglia states ion the above is that there is great variability in the levels of PSA and the ability to test for PCa. Namely, if the patient is under 60, then the level should be lowered substantially for follow up with biopsy. Yet recall, as we have shown, the biopsy has itself a 25% failure rate to detect PCa.



1.1.1.2 Zhou Approach

The Zhou approach uses a maximum likelihood detector. Whereas the Punglia approach uses a variant of the Greenes and Begg approach, the Zhou approach is fairly direct in using a ML analysis.

| | | Test=Positive (1) | Test=Negative (0) |
|--------------------------------|-----------------------|-------------------|-------------------|
| V=1 | Diagnosis=Positive(1) | X11 | X10 |
| V=1 | Diagnosis=Negative(0) | X01 | X00 |
| V=0 (Not in Validation) | | XB1 | Xb0 |
| Total | | n1 | n0 |

Note we have in the not validated section some which have positive tests and some with negative. The question is how to assign them across the groups but to do so such that Sensitivity and Specificity are estimated. Also not that if the XB are both zero then the best we could do is to calculate the two desired variables directly from the data.

To understand Zhou we follow his simplified analysis:

Let V be the group selected and let D be the diagnosis and T the test.

Then

$$P[V = 1|D, T] = P[V = 1|T]$$

or that the selection to V is independent of diagnosis D

We want the following:

$$Se = P[T = 1|D = 1]$$

$$Sp = P[T = 0|D = 0]$$

The above defined specificity and sensitivity in classic probabilistic terms. We follow Zhou's notes accordingly:

Note :

$$Se = \frac{No(T = 1, D = 1)}{No(D = 1)}$$

In reality this is the estimate of Se

Now we assume independence of V on D the diagnosis. It may depend solely upon T the test. Thus we can write using classic probability notation the following:

$$\frac{No(V = 1, T = 1, D = 1)}{No(D = 1, T = 1)} = \frac{No(V = 1, T = 1)}{No(T = 1)}$$

or

$$P(V, T, D) = P(V, T)P(D|V, T) = P(V, T)P(D|T)$$

$$P(D, T) = P(D|T)P(T)$$

thus

$$\frac{P(V, T, D)}{P(D, T)} = \frac{P(V, T)}{P(T)}$$

Thus we can write the following using the Zhou notation we have adopted²⁰:

$$\begin{aligned} & No(D = 1, T = 1) \\ &= No(V = 1, T = 1, D = 1) \frac{No(T = 1)}{No(V = 1, T = 1)} \\ &= x_{11} \frac{n_1}{x_{11} + x_{01}} \end{aligned}$$

In a similar manner we have:

$$\begin{aligned} & No(D = 1, T = 0) \\ &= No(V = 1, T = 0, D = 1) \frac{No(T = 0)}{No(V = 1, T = 0)} \\ &= x_{10} \frac{n_0}{x_{10} + x_{00}} \end{aligned}$$

We also can determine the following:

$$No(D = 1) = x_{11} \frac{n_1}{x_{11} + x_{01}} + x_{10} \frac{n_0}{x_{10} + x_{00}}$$

We can now use these in determining Sp and Se as follows²¹:

²⁰ Note that we are proportioning the total n in the sample on a pro rata basis. This is allowable since we have made the reasonable assumption of independence on V.

²¹ It should be noted that this reduces to the standard calculation if we have no un-validated samples.

$$Se = \frac{\frac{x_{11}n_1}{x_{11} + x_{01}}}{(x_{11}n_1)/(x_{11} + x_{01}) + (x_{10}n_0)/(x_{10} + x_{00})}$$

$$Sp = \frac{\frac{x_{00}n_0}{x_{10} + x_{00}}}{(x_{01}n_1)/(x_{11} + x_{01}) + (x_{00}n_0)/(x_{10} + x_{00})}$$

These are the Begg-Greenes estimators. Also called B&G estimators. We now compare the three estimators:

| | Direct | Zhou Estimate | Punglia Estimate |
|--------------------|--------|---------------|------------------|
| Sensitivity | 66.7% | 84.0% | 44.4% |
| Specificity | 72.7% | 50.4% | 87.0% |

Note the substantial difference. The BG estimate follows from the above. The Punglia uses the logistic fill and the Direct disregards the data not validated. The Zhou approach appears to be of more credibility. However there is a substantial spread in all estimates. The only solution is to biopsy all in both groups.

There is also the case of determining the ROC, or the receiver operating characteristics. This is the plot of Sensitivity versus 1-Specificity. To do this one must look at the data and then vary the decision point and plot the result. In a more classic case, we would have chosen a metric for the decision point and then mapped out the ROC as some variation in signal to noise. In this case we have not such variant only the cutoff point, namely the PSA level for determining when to take the next step.

3.2.3 Bayesian Approach

We now look briefly at a Bayesian approach suggested by Vollmer. This has not received as much attention as the previous write ups but it has substantial merit.

Vollmer starts with the expression:

$$P[PCa|PSA > x] = \frac{P[PSA > x|PCa]P[PCa]}{P[PSA > x|PCa]P[PCa] + P[PSA > x|NoPCa]P[NoPCa]}$$

This gives the Bayes approach of determining the probability of PCa given a specific value of PSA. This can then be parameterized. The term on the left is called by Vollmer as the Positive Predictive Value, PPV.

Vollmer now reorganizes the equation as follows:

$$P[PCa|PSA > x] = \frac{1}{1 + \frac{FP (1 - P[PCa])}{Sen P[PCa]}}$$

where

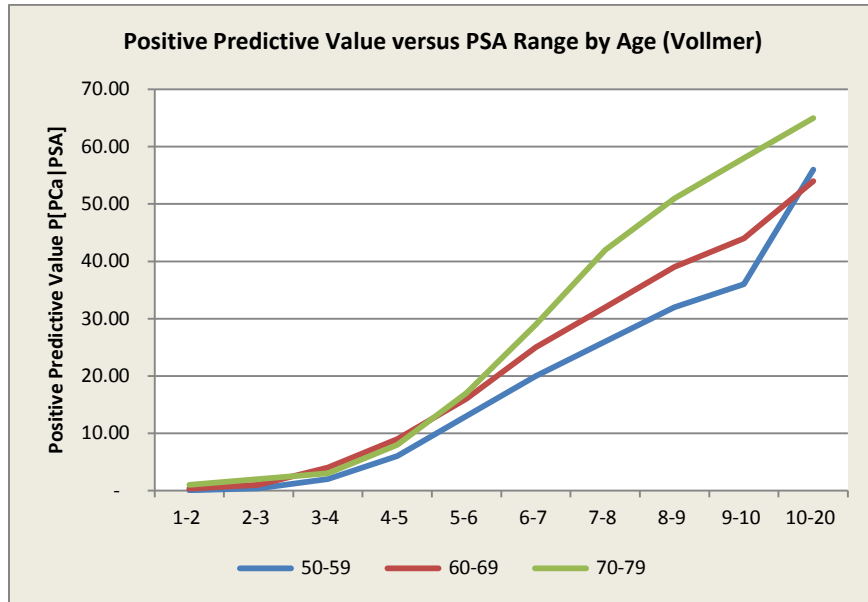
$$Sen = \text{Sensitivity} = P[PSA > x|PCa]$$

$$FP = P[PSA > x|No PCa]$$

Thus we can obtain the PPV by knowing:

1. The probability of cancer over some defined cohort
2. The sensitivity
3. The FP or false positive probability which also is 1-specificity.

Note that this approach makes some drastic assumptions. Clearly the determination of PCa over some cohort is obtainable via SEER or other types of data bases. The FP is a result of some well-defined and broad based trials. The sensitivity is also derived from many studies.



3.2.4 Summary

The above analyses has been performed to elucidate some of the techniques used to better understand the data and in turn the results of many of the clinical trials. It should be clear that despite the attempts to be as certain as one can possibly be that there are many elements of error in many of these analyses.

Verification bias is a set of methodologies to account for some of these deficiencies. In the prior section, however, we demonstrated the errors fundamental to a biopsy alone. Thus in the verification bias analysis we did not include this factor as well. Thus a zero PCa may not really

be a zero PCa, since we know that there is a finite and potentially material probability that we have missed it.

Thus we are always dealing with samples of samples. In many cases there may be an even further layer or layers of such sampling. It makes for a very complex analysis with at times questionable conclusions.

3.3 PSA DYNAMICS

Currently there are a few readily measurable factors which we can ascertain whether a patient has PCa or not. The use of PSA, PSA velocity and % Free PSA are three measures we often see used and when used they result in considerable debate. One of the issues is that the gold standard test, namely biopsy of the prostate, itself has substantial error in determining if PCa exists. The only true standard currently available is biopsy of the removed prostate. The latter gold standard is hardly one useful in clinical studies of patients with no overt signs upon normal evaluation.

We thus are looking at other measures for ascertaining patient status regarding the PCa presence. Clearly if we had a better understanding of cellular pathways and if in so understanding there were more useful markers which could be readily available, then perhaps we could have a more robust set of tests. However, lacking such, we are left with the tree measures and other exogenous variable such as age, family history, race. In this paper we use these factors as a means to ascertain the efficacy of various approaches to determining if the patient has PCa. This is not a staging approach, it is merely a monitoring effort, a screening effort, which could be used assuming that long term consistent data is available. The latter point is often a handicap since the assays used over some period of time are often highly variable in their results. We model this with a noisy measurement variable.

We thus analyze several various approaches with a primary focus on a systems approach. The systems approach is consistent with the Dougherty dictates which we try to adhere to, with predictability and reproducibility being the dominant ones. The system model we develop herein is a simple model based upon measurable parameters which can be validated by its predictable capacity. The approach is to view the resulting data such as PSA over time to be capable of providing, along with other data, more reliable metrics for assessing the potential for PCa.

The key risk in such a model is the ability to use measurable parameters across some wide base of patients. There is not reliable answer to this at the current time. Perhaps this is just a problem of “kicking the ball down the street” with solving one part of the problem by merely placing the uncertainty on another portion.

3.3.1 The Problem

We present a simple model of the problem herein. We look at the study but Punglia et al as a baseline upon which to understand the issue. We also look at the analysis we have performed regarding the probability of missing a PCa on a biopsy, which is not inconsequential.

Let us look at a simple version of Punglia model. We show this below:

| | Test Positive | Test Negative | |
|------------------|---------------|---------------|-----|
| Disease Positive | 100 | 50 | 150 |
| Disease Negative | 50 | 250 | 300 |
| | 150 | 300 | 450 |

This simple model then gives the probabilities of Sensitivity as 100/150 and Specificity of 250/300. However we know that if there were a PCa, then depending on its size we would expect a P[Missing PCa] of 25% or somewhere in that range. The question then is how does one modify this Table to account for that. Punglia modifies it for verification bias, namely just filling in those who were tested but not biopsied in some rational manner. Punglia alleges used data predicated on patient statistics. The approach was unfortunately not detailed in the paper. We performed another analysis wherein we looked at using the Zhou analysis based on Begg and Greenes approach. The answers were dramatically different.

Now using the above we get a Sensitivity of 66% and a Specificity of 83%. But let us make a simple set of assumptions for this case. We will arbitrarily assume that the miss rate in the case where there is PCa is 40% and where there is “no PCa” say 10%. The Table changes as follows:

| | Test Positive | Test Negative | |
|------------------|---------------|---------------|-----|
| Disease Positive | 130 | 20 | 150 |
| Disease Negative | 75 | 225 | 300 |
| | 205 | 245 | 450 |

Then we have:

$$\text{Sensitivity} = 130/150 = 87\%$$

$$\text{Specificity} = 225/300 = 75\%$$

| | Punglia | Adjusted |
|-------------|---------|----------|
| Sensitivity | 66% | 87% |
| Specificity | 83% | 75% |

This is a non-trivial difference. The test becomes much more sensitive. It loses some specificity but it more than makes up for sensitivity. Thus is if we were to place costs on a test and its follow up, the higher the sensitivity the better it is since we then end up treating the disease. Thus there is a need to better adjust the tests accordingly.

There are two fold elements in adjusting tests. First we desire a better test using PSA and its adjuncts. Second, we need a better way to assess the gold standard, and if no possible then adjust the data to reflect the known lack of accuracy as we have shown here.

3.3.2 Alternatives

There are many ways in which one may use the available data and then use it to ascertain the presence or absence of PCa. None have superb diagnostic characteristics as far as detections systems go. However we look at two classic approaches herein and we first introduce the systems model which we have not observed in any of the current literature. The three approaches are:

Systems Model: This is a model which looks at cell growth and the resulting markers that such cells produce. We can measure the markers such as PSA and we can ascertain experimentally all of the parameters in the model. As we have stated before, the risk is that the parameters in the model have so great a patient to patient variability that the ultimate model is of little use. However there is not adequate data at this time to make that judgment.

3.3.3 The System Approach

The systems model looks directly at the cell growth and the resulting process within cells to emit PSA into the blood stream for monitoring. We use a simple birth-death model as a first approximation for cell size.

Let assume we have a certain number of benign prostate cells. For the purpose of further simplicity we shall focus on luminal and basal cells and for the further purpose we shall use a Goldstein model and assume that luminal are derived from basal and thus can be considered as one type. Thus we assume the prostate a simply an organized collection of a single set of benign cells. Then we have:

$$\frac{dN_{Benign}(t)}{dt} = \lambda_{Benign}N_{Benign}(t) - \mu_{Benign}N_{Benign}(t)$$

$\lambda = \text{Birth Rate}$

$\mu = \text{Death Rate}$

$N = \text{Number of cells}$

Now if the cells are stable then we have birth and death rates equal. Death in this cases is by normal apoptosis and birth is mitosis. We must recall that even in mitotic growth the apoptotic process is such as to keep total cell numbers at constant levels. This in benign conditions we have:

$$N_{Benign}(t) = N_B(t) = N_B(t_0)$$

Now let us consider an amalgam of the following types of cells:

1. Benign
2. Cancer

3. PIN
4. BPH

Each has its own growth characteristics. Each has its own birth-death equations, measurable in vitro for example. Yet they may actually interact. For example PCa cells may cause increased apoptosis amongst Benign cells, pushing them aside for their own benefit. BPH may grow on top of normal cells, for in fact they are a basic extension thereto. PIN may also extend on top of Benign cells but just enlarging the prostate as would be seen with BPH but with cells confined to the glands but with differing characteristics. Thus we seek to have models which combine all. Birth and death rates may be dependent in some general way on each other. Thus we could in general posit:

$$\frac{dN_i(t)}{dt} = \left[\sum_{n=1}^6 \lambda_i(N_1, \dots, N_6) - \mu_i(N_1, \dots, N_6) \right] N_i(t) + w_i(t)$$

where

$N_1 = \text{Benign}$

$N_2 = \text{PCa}$

$N_3 = \text{PIN}$

$N_4 = \text{BPH}$

Here we have added a random process, w , which we shall assume is Gaussian Wiener process with zero mean and some determinable variance. The birth and death rates are determinable via experimental analyses.

We shall consider some simple binary models for this analysis.

Now we also note that we can relate PSA and % Free PSA (“PFP”) as functions of N , the number of specific cells. Let us consider this as follows:

$$PSA(t) = \sum_{n=1}^6 psa_n N_n(t)$$

where

$psa_n = \text{the PSA per cell of type } n \text{ in circulation}$

and

$$PPP(t) = \sum_{n=1}^6 pfp_n N_n(t)$$

where

$pfp_n = \text{the PFP per cell of type } n \text{ in circulation}$

Thus we measure PSA(t) and PFP(t) over some set of time intervals. A simple thought experiments indicates that we can see stable PSA and PFP if we have benign cells, subject to normal noise which we have included.

Let us now consider two cases.

Case I: Benign and PIN. Here we assume benign and PIN. The PIN is additional cell growth but not as extensive as say BPH. We have the following model:

$$\frac{dN_B(t)}{dt} = [\lambda_B - \mu_B] N_B(t) + w_B(t)$$

$$\frac{dN_{PIN}(t)}{dt} = [\lambda_{PIN} - \mu_{PIN}] N_{PIN}(t) + w_{PIN}(t)$$

where

$$\lambda_B - \mu_B = 0$$

$$\lambda_{PIN} - \mu_{PIN} > 0$$

Note that we stable Benign calls but a slowly growing PIN set of cells. And this yields for the exogenous measurements the following:

$$PSA(t) = psa_B N_B(t) + psa_{PIN} N_{PIN}(t)$$

and

$$PFP(t) = \frac{pfp_B N_B(t) + pfp_{PIN} N_{PIN}(t)}{N_B(t) + N_{PIN}(t)}$$

Now as we see more PIN cells we see a slowly increasing PSA, subject to noise, and we see a PPT also changing on a weighted basis. Yet if pfb is identical for both Benign and PIN then we see that PFP remains constant and high.

Case II: PCa: In this case we have benign and cancer cells. The same model as above but with some substantial modifications. We see this first as follows:

$$\frac{dN_B(t)}{dt} = [\lambda_B - \mu_B(N_{PCa}(t))] N_B(t) + w_B(t)$$

$$\frac{dN_{PCa}(t)}{dt} = [\lambda_{PCa} - \mu_{PCa}] N_{PCa}(t) + w_{PCa}(t)$$

where

$$\lambda_B - \mu_B > 0$$

$$\lambda_{PCa} - \mu_{PCa} \gg 0$$

and

$$\frac{\partial \mu_B}{\partial N_{PCa}} > 0$$

This implies that we have a decreasing cell count of benign cells and an increasing and growing count of PCa cells. Thus when we calculate the following:

$$PSA(t) = psa_B N_B(t) + psa_{PCa} N_{PCa}(t)$$

and

$$PFP(t) = \frac{pfp_B N_B(t) + pfp_{PCa} N_{PCa}(t)}{N_B(t) + N_{PCa}(t)}$$

We see that the number of PCa cells are growing and at a rate in excess of and Benign cells, which are declining and that psa of PCa is much smaller than that of Benign cells as it the pfp of PCa, which is quite small as compared to benign cells. Thus with PCa we see PSA increasing and PFP decreasing.

Now the question we pose is how do we determine:

$$P[PCa | PSA(s), PFP(s); s \in (t_0, t)]$$

This is a classic detection problem. We have solved that problem in our earlier work²². We will present the analytical approach here. Before continuing, however, we want to demonstrate what we know and what we have speculated:

We know the following from experiment and can validate from more experiments:

1. Cell growth follows the models we have depicted.
2. Growth rates are determinable from such factors as mitotic rates and other methods which are well known.
3. Cancer cells do push our benign cells through a variety of methods which are well understood.
4. The measurements we have determined are well documented and the average rates we use in the models are determinable from measurements.

We do not really know the following:

1. The functional characteristic of the increased death rate, and even birth rate, of benign cells dependent on the new PCa cells. There is the issue of the PCa cells absorbing nutrients from the Benign cells as well as the issue of reducing normal mitotic reactions.

3.3.4 Hypothesis Detection Model

The detection model can be defined as follows:

Hypothesis 0: Benign

²² See McGarty, *Stochastic Systems and State Estimation*, Wiley, 1974.

$$PSA(t) = psa_B N_B(t)$$

and

$$PFP(t) = \frac{pfp_B N_B(t)}{N_B(t)}$$

And

$$\frac{dN_B(t)}{dt} = [\lambda_B - \mu_B] N_B(t) + w_B(t)$$

where

$$\lambda_B - \mu_B = 0$$

Hypothesis 1: PCa

$$PSA(t) = psa_B N_B(t) + psa_{PCa} N_{PCa}(t)$$

and

$$PFP(t) = \frac{pfp_B N_B(t) + pfp_{PCa} N_{PCa}(t)}{N_B(t) + N_{PCa}(t)}$$

and

$$\frac{dN_B(t)}{dt} = [\lambda_B - \mu_B(N_{PCa}(t))] N_B(t) + w_B(t)$$

$$\frac{dN_{PCa}(t)}{dt} = [\lambda_{PCa} - \mu_{PCa}] N_{PCa}(t) + w_{PCa}(t)$$

where

$$\lambda_B - \mu_B > 0$$

$$\lambda_{PCa} - \mu_{PCa} \gg 0$$

and

$$\frac{\partial \mu_B}{\partial N_{PCa}} > 0$$

Thus we want to find a detector, maximum likelihood as an example, using:

$$P[PCa|DataSet] = \frac{P[DataSet|PCa]P[PCa]}{P[DataSet]}$$

3.3.5 Adequacy of Data in Model

We now take a brief look at what the effects of patient to patient variability would be in the model. As we said, there are measurable constants which we can ascertain and use in the model. There are two sets of the constants. The first set if the growth parameters and the second is the measurement parameters.

Let us consider the growth first. We assume that there is an average parameter and some variation about that average. We then ask how do we modify the model accordingly. This is a simple first order modification where the δ represent the zero mean variation of the measurement of the related variable with a variance σ associated with it as determined from the measurement data. Thus we have:

$$\begin{aligned}\frac{dN_B(t)}{dt} &= [\hat{\lambda}_B + \delta\lambda_B - \hat{\mu}_B + \delta\mu_B] N_B(t) + w_B(t) \\ &= [\hat{\lambda}_B - \hat{\mu}_B] N_B(t) + [\delta\lambda_B + \delta\mu_B] N_B(t) + w(t) \\ &= [\hat{\lambda}_B - \hat{\mu}_B] N_B(t) + u(t) + w(t)\end{aligned}$$

where

$$\lambda_B - \mu_B = 0$$

This model then uses the uncertainty of the measurements as an added noise term, albeit correlated with the cell count. If the “noise” associated with the measurements is small with respect to the count itself then we can reasonably augment the overall system noise to include that level.

This is a first order approach to including the issue of measurement uncertainty of the underlying parameters.

We can do the same with the measurements:

$$\begin{aligned}PSA(t) &= psa_B N_B(t) + psa_{PCa} N_{PCa}(t) \\ &= (psa_B) N_B(t) + ps\hat{a}_{PCa} N_{PCa}(t) + \delta psa_B N_B(t) + \delta ps\hat{a}_{PCa} N_{PCa}(t) \\ &= (ps\hat{a}_B) N_B(t) + ps\hat{a}_{PCa} N_{PCa}(t) + r(t)\end{aligned}$$

Where we replace the uncertainty with an $r(t)$ as we did above.

3.4 LOGISTIC ANALYSES

The logistic approach looks at the probability of PCa and its dependence on certain variables. For the purpose of this analysis we know that it depends on:

1. PSA Level
2. % Free PSA
3. Velocity of PSA

4. Age
5. First Degree Relatives Having PCa
6. Race

This in a simple logistic model we define:

$$\ln \left[\frac{P[PCa]}{1 - P[PCa]} \right] = \alpha + \sum_{i=1}^6 \beta_i x_i$$

where

$x_1 = PSA\ level$

$x_2 = \% Free\ PSA$

$x_3 = PSA\ velocity$

$x_4 = Age$

$x_5 = First\ Degree\ Relatives$

$x_6 = Race$

As compared to the system model which is based upon verifiable constants and an clear underlying physical process and model, this is pure statistical conjecture. Here we will use volumes of data to attempt to ascertain the relationships. In logistic analysis the relationship is posited ab initio and there may or may not be any underlying physical relationship. We merely use the data and then from the data try to fit the constants based upon a clinical determination of the disease state.

3.5 CLASSIFICATION METHODOLOGIES

Classification approaches include such methods as clustering, principal component analyses, and other such methods. If we have say six measurables at our hand then we can collect a great deal of data with an assumed determination of PCa being absent or present. Then in this six dimensional space we can map out sectors which show how we could split the space into PCa and Benign space. We leave it to the reader to see the use of these techniques and refer them to the references at the end of this paper. As Dougherty so aptly states, the use of many classifiers are based solely upon the data and its characteristics and it devoid of any understanding of the inherent pathology.

3.6 A MAXIMUM LIKELIHOOD SYSTEMS CLASSIFIER

We can now use the systems model to develop a classifier. We start with a simple binary decision between two hypotheses; benign or PCa. We assume that the system can be delivered in a discrete time manner, which frankly we know. We will follow the approach in VanTrees for this analysis. Thus we have for the system:

$$N_B(k+1) = N_B(k) + (\lambda_B(k) - \mu_B(k))N_B(k) + w_B(k)$$

under H_0 which is the hypothesis of benign

and under this hypothesis we have

$$N(k) = N_B(k)$$

$$N_B(k+1) = N_B(k) + (\lambda_B(k) - \mu_B(k))N_B(k) + w_B(k)$$

$$N_{PCa}(k+1) = N_{PCa}(k) + (\lambda_{PCa}(k) - \mu_{PCa}(k))N_{PCa}(k) + w_{PCa}(k)$$

under H_1 which is the hypothesis of PCa

and under H_1 we have

$$N(k) = N_B(k) + N_{PCa}(k)$$

This is a model for a Markov process assuming the noise is independent and Gaussian and it has zero mean. The variance may be time or sample dependent. Note also that we may have to adjust the birth and death constants to reflect the time between samples.

Now what we measure is:

Under H_0 we have:

$$PSA(k) = psa_B N_B(k) + n_{PSA,B}(k)$$

and

$$PFP(k) = \frac{pfp_B N_B(k)}{N_B(k)} = pfb_B N_B(k) + n_{PFP,B}(k)$$

Under H_1 we have:

$$PSA(k) = psa_B N_B(k) + psa_{PCa} N_{PCa}(k) + n_{PSA,Both}(k)$$

and

$$PFP(k) = \frac{pfp_B N_B(k) + pfp_{PCa} N_{PCa}(k)}{N_B(k) + N_{PCa}(k)} + n_{PFP,Both}(k)$$

Here the $n(k)$ is a measurement noise sequence reflecting both assay errors as well as variations from the base line estimates. What we use for the decision statistics are the above sets of variables. The difficulty would be that they are derived from the same data sequences, the $N(k)$ sequences and thus are combinations of variables. Also we can simplify the PFP by normalizing it by volume, assuming that the cells are each of equal volume. Namely benign cells and PCa cell have essentially the same volume. Thus we can write the above measurements as a simplified linear model as follows:

Under H_0 we have:

$$PSA(k) = psa_B N_B(k) + n_{PSA,B}(k)$$

and

$$PFP(k) = pfb_B N_B(k) + n_{PFP,B}(k)$$

Under H_1 we have:

$$PSA(k) = psa_B N_B(k) + psa_{PCa} N_{PCa}(k) + n_{PSA,Both}(k)$$

and

$$PFP(k) = pfp_B N_B(k) + pfp_{PCa} N_{PCa}(k) + n_{PFP,Both}(k)$$

Where we use volumetric normalized values for PFP.

Now we want the probabilities of PSA and PFP for all ks. We can write²³:

For H_0

$$p(PSA(k), PFP(k) | N(k)) = \tilde{N}(psaN(k), \sigma_{PSA}) \tilde{N}(pfpN(k), \sigma_{PFP})$$

and

$$p(PSA(k), PFP(k), N(k) | N(k-1)) = \tilde{N}(psaN(k), \sigma_{PSA}) \tilde{N}(pfpN(k), \sigma_{PFP}) \tilde{N}((\lambda - \mu)N(k-1), \sigma_N(k))$$

Thus we have the joint conditional probability being all Gaussian with known means and we know that the $N(k)$ s are themselves incrementally conditionally independent since we have a Wiener process and it is independent.

Now if we use the likelihood ratio we want the following:

Let

$$r_{PSA} = \begin{bmatrix} PSA(1) \\ \dots \\ PSA(n) \end{bmatrix}$$

$$r_{PFP} = \begin{bmatrix} PFP(1) \\ \dots \\ PFP(n) \end{bmatrix}$$

$$r = \begin{bmatrix} r_{PSA} \\ r_{PFP} \end{bmatrix}$$

These represent the received vectors. To define the likelihood ratios we then use these:

²³ Note we use the notation $N(a,b)$ as a normal or Gaussian distribution with mean a and standard deviation b .

$$p(r|H_0) = \int p(r|x, H_0)p(x|H_0)dx$$

But

$$p(r|x, H_0) = \prod_{n=1}^N p(r_n|x_n, H_0)$$

and

$$p(x|H_0) = \prod_{n=1}^N p(x_n|x_{n-1}, H_0)$$

And they are all normal with defined means and variances. We thus can pairwise deal with these. However the inclusion of noise on the cell count model adds a bit of complexity so we shall assume that it can be ignore in a first order approximation. Then we can easily determine the likelihood ratio parameters as follows:

For H_0

$$N_B(k+1) = N_B(k) + (\lambda - \mu)N_B(k)$$

and for non-uniform intervals we write:

$$N_B(k+1) = N_B(k) + (\lambda - \mu)\Delta(k)N_B(k)$$

where we have λ and μ normalized accordingly

$\Delta(k)$ then is the sample time difference

For the measurements we have:

$$PSA(k) = psa_B N_B(k) + n_{PSA,B}(k)$$

and

$$PFP(k) = pfb_B N_B(k) + n_{PFP,B}(k)$$

These are independent random variables driven by the underlying count. Note that the sampling time issues plays no part in this expression. Obviously we have the same for the other case of PCa.

It can easily be shown that the likelihood ratio, specifically the log likelihood ratio can be given as follows:

Choose H_0 if:

$$\sum_{n=1}^N \left[PSA(k) - psa_B \alpha_B \Delta(n) N_0 \right]^2 + \left[PFP(k) - pfp_B \alpha_B \Delta(n) N_0 \right]^2 >$$

$$\sum_{n=1}^N \frac{\left[PSA(k) - psa_{B,PCa} \alpha_{B,PCa} \Delta(n) N_0 - psa_{PCa} \alpha_{PCa} \Delta(n) N_0^{PCa} \right]^2}{\sigma_{PSA}^2}$$

$$+ \frac{\left[PFP(k) - pfp_{B,PCa} \alpha_{B,PCa} \Delta(n) N_0 - pfp_{PCa} \alpha_{PCa} \Delta(n) N_0^{PCa} \right]^2}{\sigma_{PFP}^2}$$

Now we can consider the issue of choosing between the four hypotheses; B, PIN, BPH, and PCa. Again we rely upon the treatment in VanTrees. The model follows directly from above.

Let the following be the hypotheses:

$$H_0 = \text{Benign}$$

$$H_1 = \text{BPH}$$

$$H_2 = \text{PIN}$$

$$H_3 = \text{PCa}$$

Then we create the following likelihood ratios:

$$\Lambda_{i,j}(r) = \frac{p(r|H_i)}{p(r|H_j)}$$

Then we can set up the decision regions based upon the following rules:

$$\Lambda_{0,1}(r) \begin{cases} < c_{0,1} : \text{Choose } H_0 \text{ or } H_2 \text{ or } H_3 \\ > c_{0,1} : \text{Choose } H_1 \text{ or } H_2 \text{ or } H_3 \end{cases}$$

$$\Lambda_{1,2}(r) \begin{cases} < c_{1,2} : \text{Choose } H_1 \text{ or } H_0 \text{ or } H_3 \\ > c_{1,2} : \text{Choose } H_2 \text{ or } H_0 \text{ or } H_3 \end{cases}$$

$$\Lambda_{2,3}(r) \begin{cases} < c_{2,3} : \text{Choose } H_2 \text{ or } H_0 \text{ or } H_1 \\ > c_{2,3} : \text{Choose } H_3 \text{ or } H_0 \text{ or } H_1 \end{cases}$$

$$\Lambda_{0,2}(r) \begin{cases} < c_{0,2} : \text{Choose } H_0 \text{ or } H_1 \text{ or } H_3 \\ > c_{0,2} : \text{Choose } H_2 \text{ or } H_1 \text{ or } H_3 \end{cases}$$

$$\Lambda_{0,3}(r) \begin{cases} < c_{0,3} : \text{Choose } H_0 \text{ or } H_1 \text{ or } H_2 \\ > c_{0,3} : \text{Choose } H_3 \text{ or } H_1 \text{ or } H_2 \end{cases}$$

$$\Lambda_{1,3}(r) \begin{cases} < c_{1,3} : \text{Choose } H_1 \text{ or } H_0 \text{ or } H_2 \\ > c_{1,3} : \text{Choose } H_3 \text{ or } H_0 \text{ or } H_2 \end{cases}$$

These then set out mutually exclusive decision regions. The details are in VanTrees. Generally we seek a binary decision between something and PCa. Knowing these regions we can quantitatively calculate the ROC related probabilities and we can choose the thresholds to maximize the ROC areas as has been suggested in the literature.

3.7 EXAMPLE

We now consider a simple example. This is one where we are looking at almost 20 years of data, some missing, and we then look at a binary hypothesis of B or PIN. Consider the data on the following patient:

| Year | PSA(Alone) | Delta PSA | Delta/Yr PSA Abs | PSA Velocity 3-SampleTests | PSA Free | PSA on Free PSA | %Free PSA |
|--------|------------|-----------|---------------------|-------------------------------|----------|--------------------|-----------|
| Feb-93 | 0.62 | - | - | - | - | - | |
| Mar-94 | 0.53 | (0.15) | (0.09) | - | - | - | |
| Feb-95 | 1.50 | 1.76 | 1.01 | - | - | - | |
| Jan-96 | 0.62 | (0.53) | (0.98) | (0.02) | - | - | |
| Jan-97 | 0.70 | 0.13 | 0.08 | 0.04 | - | - | |
| Apr-98 | 0.77 | 0.12 | 0.06 | (0.28) | - | - | |
| Aug-99 | 0.95 | 0.31 | 0.14 | 0.09 | - | - | |
| Jul-00 | 1.10 | 0.14 | 0.16 | 0.12 | - | - | |
| Aug-00 | 1.10 | - | - | 0.10 | - | - | |
| Oct-01 | 1.10 | - | - | 0.05 | - | - | |
| Nov-02 | 1.30 | 0.19 | 0.19 | 0.06 | - | - | |
| Nov-03 | 1.19 | (0.08) | (0.11) | 0.03 | 0.50 | 1.30 | 38% |
| Nov-04 | 1.53 | 0.30 | 0.32 | 0.13 | 0.50 | 1.53 | 33% |
| Nov-05 | 1.22 | (0.19) | (0.33) | (0.04) | 0.50 | 1.53 | 33% |
| Dec-06 | 1.60 | 0.35 | 0.34 | 0.11 | 0.50 | 1.53 | 33% |
| Nov-07 | 1.49 | (0.06) | (0.12) | (0.04) | 0.50 | 1.53 | 33% |
| Nov-08 | 1.49 | - | - | 0.07 | - | - | |
| Nov-09 | 2.20 | 0.48 | 0.70 | 0.19 | - | - | |
| Feb-10 | 2.10 | (0.01) | (0.52) | 0.06 | 0.70 | 1.53 | 33% |
| Feb-10 | 1.80 | (0.00) | (2.03) | (0.62) | 0.70 | 1.53 | 39% |
| May-10 | 1.70 | (0.01) | (1.57) | (1.37) | 0.70 | 1.53 | 39% |
| Oct-10 | 2.00 | 0.08 | 0.27 | (1.11) | - | - | |

We now use the test we had above. We must look at the underlying statistics.

1. Variance of both PSA and PFP are about a 25% standard deviation. Thus since both are the same these factors can be removed from the analysis.
2. The number of normal cells in a 40 cc prostate can be assumed to be 10 million. We assume that we can normalize cell numbers in millions so that a cell count of 10 is the equivalent of 10 million.
3. We can assume that a benign prostate of 40 cc has a base level in a 40 year old male is 0.5 and PFP is 35%.

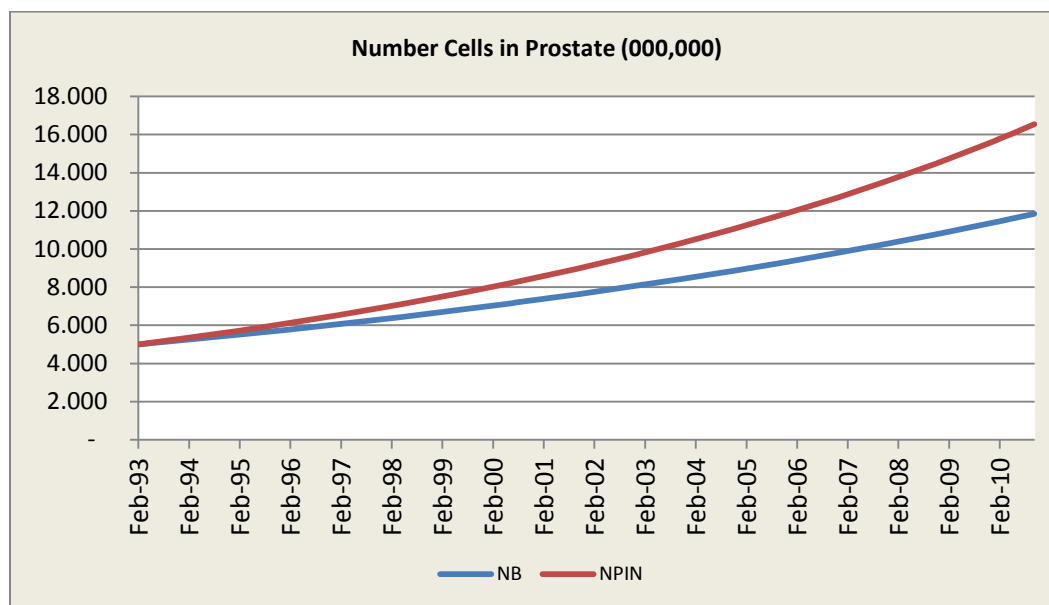
4. We can further assume that we have in a normal prostate a 25% increase in size per decade as the man ages over 50. Thus there is a 25% change. In contrast with BPH the doubling is every 5 years and for PIN we have every 7.5.

5. We assume that both BPH and PIN cells secrete the same PSA and the binding is the same.

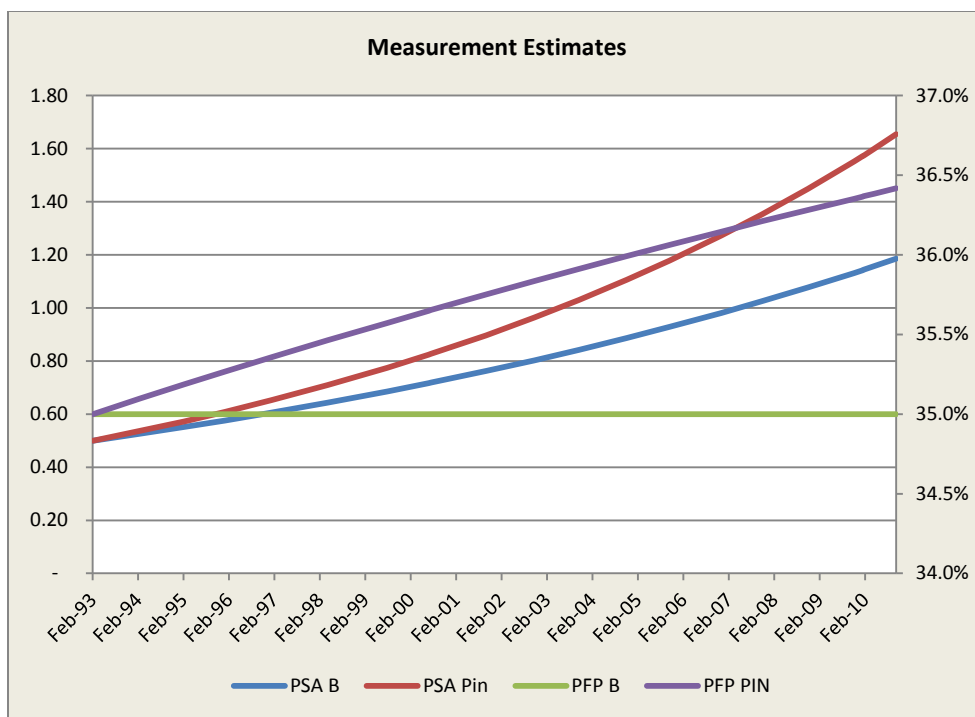
6. We assume that the doubling rate for cells with PCa is much shorter, namely 3 months and that PSA is the same per cell but PFP is 5% per cell not 35%.

The next issue is to establish a baseline for the incidence of any of these states, namely when do we measure X_0 . For simplicity we assume at 50 that all X_0 are the same, based on a 40 year old baseline. This is one of the concerns with this model, namely establishing a baseline. We argue that similar estimation techniques can provide that as well.

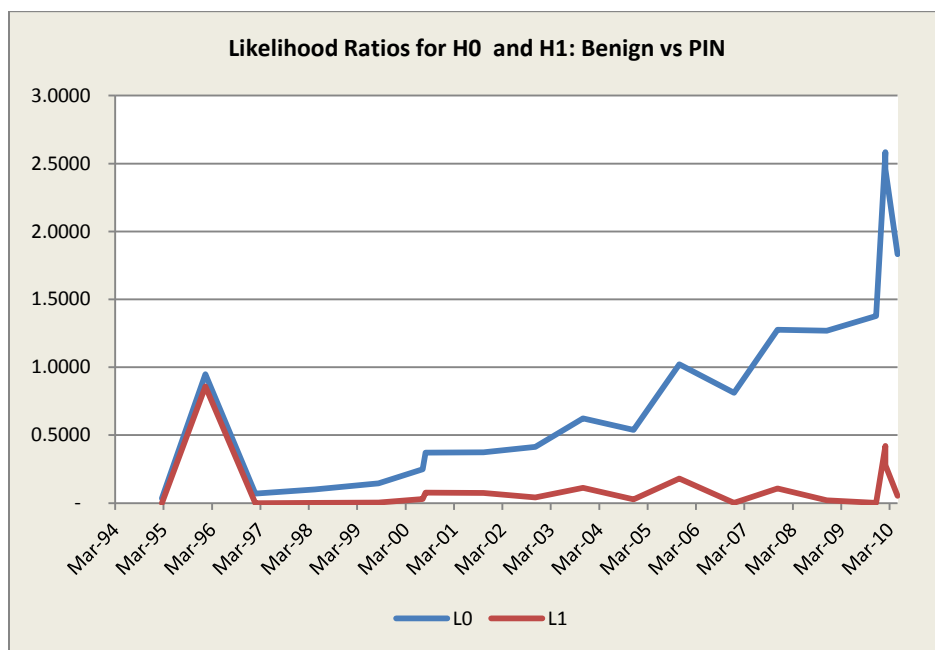
We now use this on the data we have shown earlier. First we show the cell growth under two assumptions:



Then we show the projected measurement values to be used against the real measurements.



Then we show the likelihood ratios. Remember the selection is the smallest value based on it yielding the largest likelihood.



The interesting metric is the fact that we have a growing likelihood that the data suggests even five years earlier that PIN was present.

Thus we have shown that this maximum likelihood approach as modified appears to be readily applied and provides a strong suggestive set of guidelines for the physician.

3.8 CONCLUSIONS

We have developed an alternative approach to the use of the limited data for assessing the risk of PCa in patients. It is an approach which is based upon the underlying dynamics of the cellular system and reflects the impact of key parameters of different cell growth rate and their impact on the measured variables. We have also shown that

1. The new metric requires a long period of collecting data on PSA and PFP. It then requires having reliable data on growth in the four differing scenarios. However it is interesting in that by including the data in this form we are effectively including velocity data implicitly.
2. The underlying constants may be based upon other factors as well, namely race, family history, and age. The Punglia paper does look somewhat at age segregation and recommends lower thresholds. We argue here that a running statistic may provide an improved discriminant.
3. ROC characteristics can be calculated analytically from this approach assuming certain constants.
4. The approach is direct and simple and seems to allow for early detection via a tracking of the likelihood ratio.

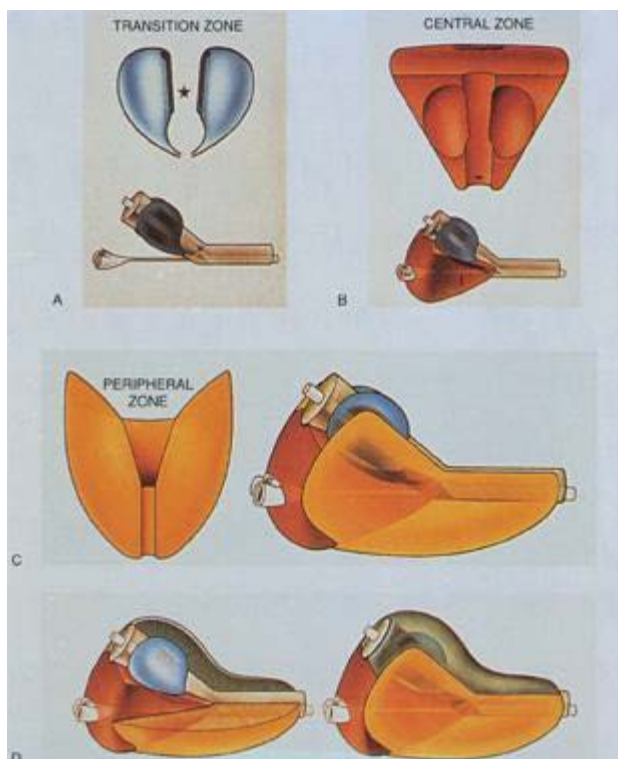
4 PROSTATE HISTOLOGY AND PATHOLOGY

In this Chapter we examine first the normal histology of the prostate and then we examine various types of dysplasia and malignancies. The intent here is not to become expert in the histological specificities of the prostate in both benign and malignant state but to have a fundamental understanding of how on a microscopic scale a malignancy develops and progresses. This will then allow us to, on the one hand look deeper into the genetic mechanism, and on the other hand, be able to look upward to cancer as a system level disease. The ultimate objective is to develop that system model for prostate cancer which aligns with the genetic underpinnings as well as being reflective of the histological development.

4.1 THE NORMAL PROSTATE

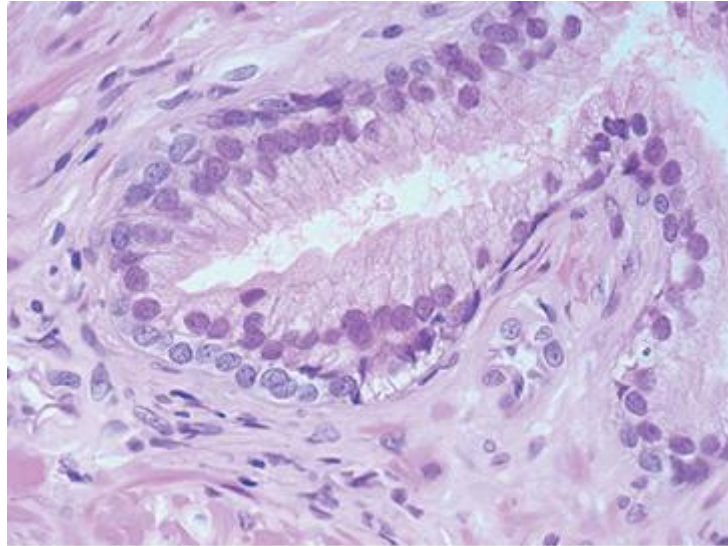
We first examine the normal prostate. The prostate is normally about 40 cc in dimension with the prostate surrounding the urethra below the bladder.

The basic structure of the prostate is shown below. It consists of three major zones; peripheral (dominant zone), central zone which is around the urethra, and the transition zone.

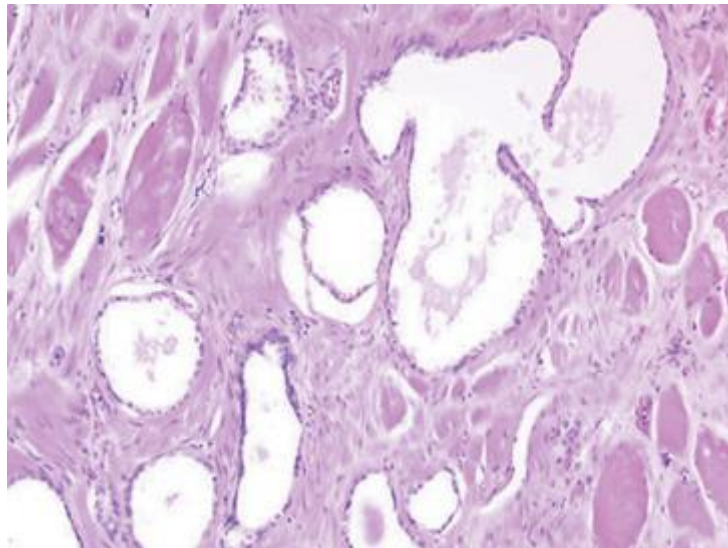


The cellular structure is depicted below. There are approximately 35-50 glands in the prostate, mostly in the peripheral zone and the glands have a lumen in which the prostatic secretions flow and the glands have basal cells and luminal cells as shown below. The basal cells are dark and the luminal cells are somewhat lighter.

Between the cells is the stroma which includes the blood flow from veins and arteries, the muscle and other stroma elements. Simply stated, the prostate is a collection of the basal/luminal glands scattered about veins, arteries, muscles and nerves.



The figure below depicts a second view of the prostate glands. Again this is with HE stain and under low magnification. The basal cells are clearly see with their dark stains and the luminal stand above them. The stroma is fairly well articulated in this slide.

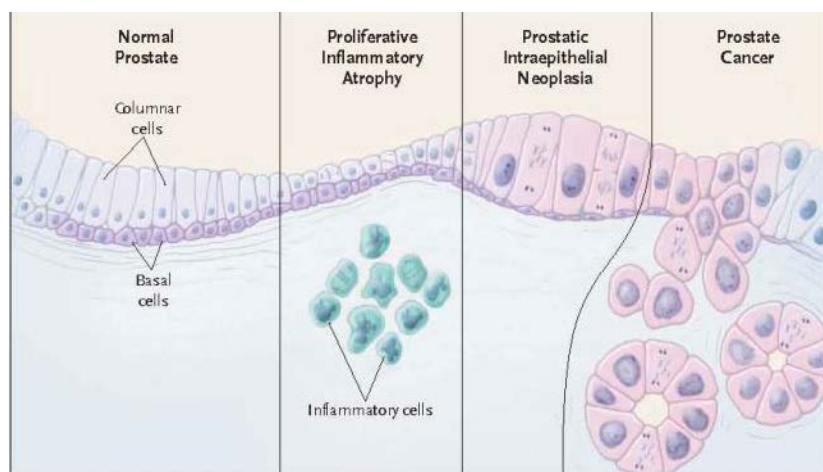


The normal prostate then is merely a collection of glands, glands composed of basal and luminal cells, with open glandular portions, the white areas above. As we noted before these glands emit various proteins and are an integral part of the male reproductive system.

4.2 SUMMARY OF PROSTATE STATES

We now provide a high level summary of the changes in the prostate histologically as PCa is developed. We do this to lay out the various changes we will examine and to better understand what we may be looking for when developing pathways. We believe that it is essential that we always go back and forth between abstractions of pathways, and the reality of the cell histology.


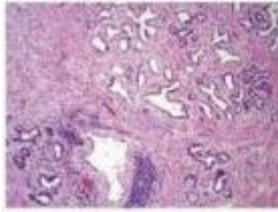

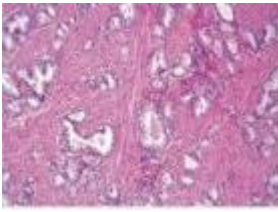
There is a general agreement, with of course many exceptions, as to the progression of prostate pathology and its related causes. A graphic from a recent NEJM article is shown below²⁴:

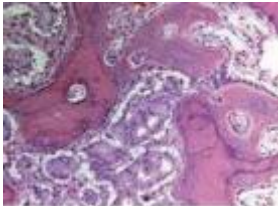



Not the progression from normal prostate with basal and luminal cells and then through PIA and then PIN and finally PCa. The PIN demonstrates a complex but contained development of cells. As one moves to PCa, that is when the cells move away from the existing gland, and they are for the most part luminal cells establishing de novo glandular like structures.

An excellent tabular summary from Taichman et al follows:

²⁴ See Nelson et al, Prostate Cancer, NEJM, July 24, 2003. p 376.

| <i>Disease State</i> | <i>Histology</i> | <i>Details</i> |
|----------------------|---|--|
| Normal Prostate |  | <p>Large glands with papillary infoldings that are lined with a 2-cell layer consisting of basal and columnary secretory epithelial cells (luminal) with pale cytoplasm and uniform nuclei.</p> <p>Susceptibility genes or events related to hereditary PCa:</p> <p>RNASEL: regulates cell proliferation through the interferon regulated 2-5 oligoadenylate pathway</p> <p>ELAC2/HPC2: Loss of function of tRNA-3 processing endoribonuclease</p> <p>MSR1: Macrophage scavenger receptors process negatively charged macromolecules.</p> |
| PIA |  | <p>Atrophic glands have scant cytoplasm, hyperchromic nuclei and occasional nucleoli and are associated with inflammation</p> <p>Susceptibility genes or events:</p> <p>NKX3: Allelic loss of homeobox protein allowing growth of prostate epithelial cells</p> <p>PTEN: Allelic loss of phosphatase and tensin homolog allowing decreased apoptosis and increased cell proliferation.</p> <p>CDKN1B: Allelic loss of cyclin dependent kinase inhibitor p27 allowing increased cell proliferation</p> |
| PIN |  | <p>Intermediate to large size glands with proliferation changes contained within the gland and having nuclear abnormalities that resemble invasive carcinoma.</p> <p>Susceptibility genes or events:</p> <p>GSTP1: Hypermethylation of the upstream regulatory region inactivates the Pi class glutathione S transferase enzyme which detoxifies carcinogens.</p> <p>Hepsin: Increased expression of this serine protease leads to increased invasiveness and disruption of the basement membrane.</p> <p>AMACR: Increased expression results in increased peroxisomal b-oxidation of branched chain fatty acids from red meat thereby increasing carcinogen exposure.</p> <p>TMPRSS2: Fusion of this androgen regulated gene with ETS family of transcription factors in late stages of PIN results in increased breakdown of the extracellular matrix.</p> <p>Telomerase: Activation leads to maintenance of telomere length and immortalization of cells.</p> |
| Prostate Cancer |  | <p>Small irregular glands with cells having abnormal nuclei and nucleoli and lacking basal cells.</p> <p>Susceptibility genes or events:</p> <p>MYC: Overexpression leads to cell proliferation and transformation</p> <p>RB: Loss of expression leads to cell proliferation and transformation</p> |

| <i>Disease State</i> | <i>Histology</i> | <i>Details</i> |
|----------------------|---|--|
| Metastatic PCa |  | <p>Nests of cancer cells within the bone</p> <p>Susceptibility genes or events:</p> <p>TP53: Mutation results in loss of multiple tumor suppressor functions</p> <p>E-cadherin: Aberrant expression leads to increased invasive and metastatic phenotype</p> <p>NM23: loss of this NDP kinase leads to increased metastasis</p> <p>EZH2: Histone methyltransferase PcG protein whose activation causes repression of genes that suppress invasion and metastasis</p> |
| AR PCa |  | <p>Cancer cells that grow in androgen depleted environment</p> <p>Susceptibility genes or events:</p> <p>AR: may remain active through amplification, phosphorylation by other steroids or non-androgen growth factors</p> <p>BCL2 Increased expression leads to protection from apoptosis</p> <p>Stem cells: potential repopulation by progenitor cells</p> |

Note in the above, Taichman et al make mention of the separate gene elements that are putatively assumed to have caused the subsequent event. These genetic changes then will become a key factor in how we view PIN transitions.

Also note in the above, it implies a set of sequences of genetic changes that moves from benign to malignant. The question then is; if a genetic change is necessary for a morphological change, then is the genetic change reversible or are the genetically changed cells killed off by some other process, and if so what process?

To understand this question, and hopefully set a path to answering it, we lay out the known elements in the path towards malignancy, look at the gene maps and dynamics, and then attempt to establish a model for examining the dynamic processes which move the cell forward to malignancy or backwards towards a benign state.

We shall now examine each of these in some detail.

4.3 PROSTATIC INTRAEPITHELIAL NEOPLASIA

Prostatic Intraepithelial Neoplasia, PIN, is considered a precursor to PCa. High Grade PIN, HGPIN, is often considered almost certainly a precursor. However as we shall discuss this is at times not the case and HGPIN is known to regress. One must be careful, however, since we are generally discussing biopsy samples which may be subject to substantial sampling deficiencies as we have already discussed.

Let us now provide a simple overview of the development of models. We develop it in the following manner:

First, we look at the histological structure of PIN and PCa. Cell changes occur and the changes morphologically are dependent upon the expression of or lack thereof of certain genes. The

linking of morphology and gene expressions seems to fall short at this stage. Thus the nexus is missing.

Second, we look at some simple models for the development of HGPIN. As we have stated, the reason for this is twofold. First HGPIN is often assumed to be a natural precursor of PCa and as such one can assume that genetic changes necessary for PCa are first seen in HGPIN. Second we know that HGPIN can suddenly regress and the cells revert to benign state. If that is the case and indeed it is one may ask if the genetic changes were the cause also of the regression or was there some exogenous cause. We focus primarily on the Goldstein et al model because it demonstrates both HGPIN and PCa and the relationship to morphological and genetic changes.

Third, we examine the cancer stem cell, CSC, model. The CSC is an interesting paradigm which may explain the less than rapid growth of certain cancers. PCa may be dominated in many cases by indolent slow reproducing CSC. Understanding the dynamics of the CSC is therefore essential.

Fourth, we look at the many specific genetic drivers such as PTEN and the other first and second order products in the pathway chain. This is an extensive discussion which we will rely upon to build pathway models.

Fifth, we examine the epigenetic factors such as miRNA and methylation. These may be the most significant factors in cell change and genetic expression alteration that we see in PCa progression.

Sixth, we present and examine in some high level detail the many complex pathway models currently presented.

Seventh, we examine the various models for reaction kinetics. This will be essential when we attempt to model the dynamics. The classic approaches are significant and their simplifications are useful. By looking at linear models we often can find reasonable insight but it is often by examining the nonlinear models that we can ascertain the tipping points with more clarity.

Eighth, we examine pathway controls that are what components such as PTEN play the most significant role.

Ninth, we look at the three dominant modeling techniques; Boolean, Bayesian, and System model using reaction rates and complex time varying differential equations. We do not in this analysis examine the spatial models (as initially developed by Turing and detailed by Murray).

Tenth, we examine how the constants in these models may be obtained by means of system identification methods. We have accomplished this in other pathway systems and we believe it is directly applicable here as well.

4.3.1 HGPIN Characterization

HGPIN is represented by morphological changes in prostate cells in the acinar or glandular locations. It generally is a complex set of growth patterns of new cells whose morphological appearance is similar to but not identical to the existing cells in the gland. The new cells clearly have form and shape that demonstrates pre-malignant morphology, with enlarge and prominent nucleoli.

From the paper by Putzi and DeMarzo we have:

The high-grade form of prostatic intraepithelial neoplasia (PIN) has been postulated to be the precursor to peripheral zone carcinoma of the prostate. This is based on zonal co-localization, morphologic transitions, and phenotypic and molecular genetic similarities between high-grade PIN and carcinoma. Although high-grade PIN is thought to arise from low-grade PIN, which in turn is thought to arise in normal or “active” epithelium, little is known whether truly normal epithelium gives rise to PIN or whether some other lesion may be involved.

Focal atrophy of the prostate, which includes both simple atrophy and postatrophic hyperplasia, is often associated with chronic, and less frequently, acute inflammation. Unlike the type of prostatic atrophy associated with androgen withdrawal/ blockade (hormonal atrophy), epithelial cells in simple atrophy/postatrophic hyperplasia have a low frequency of apoptosis and are highly proliferative. In addition, hormonal atrophy occurs diffusely throughout the gland and is not usually associated with inflammation.

To simplify terminology and to account for the frequent association with inflammation and a high proliferative index in focal atrophy of the prostate, we introduced the term “proliferative inflammatory atrophy” (PIA).

In a similar manner in a review paper by O’Shaughnessy et al on multiple intraepithelial neoplasia the authors state the following regarding HGPIN:

The evidence that PIN is a morphological and genetic precursor to prostate cancer is extensive and conclusive...

When examined microscopically, PIN lesions are characterized by collections of proliferative prostatic epithelial cells confined within prostatic ducts that exhibit many morphological features of prostate cancer cells, including architectural disorganization, enlarged cell nuclei and nucleoli. ...

In addition to the similarity of the cellular morphologies of HGPIN and invasive lesions, evidence that HGPIN is a precursor of prostatic adenocarcinoma includes the multifocality of both lesions and the presence of carcinoma in foci of PIN; among older men, foci of PIN are found in 82% of prostates with carcinoma but in only 43% of normal prostates.

PIN is frequently located in the peripheral zone of the prostate, the site at which 70% of prostatic carcinomas occur. Additional similarities include enhanced proliferative activity of both PIN and carcinoma (3-fold that of benign tissue), cytokeratin immunoreactivity, lectin binding, and loss of blood group antigen with both PIN and carcinoma.

Prevalence of PIN and its temporal association with invasive cancer are illustrated by the known 40–50% PIN incidence in men 40–60 years of age, evolving into the 40–50% incidence of prostate cancer in men 80 years of age. Autopsy data reveal that PIN lesions appear in the prostates of men in their 20s and 30s in the United States, preceding the appearance of prostate cancer lesions by as many as 10 years ...

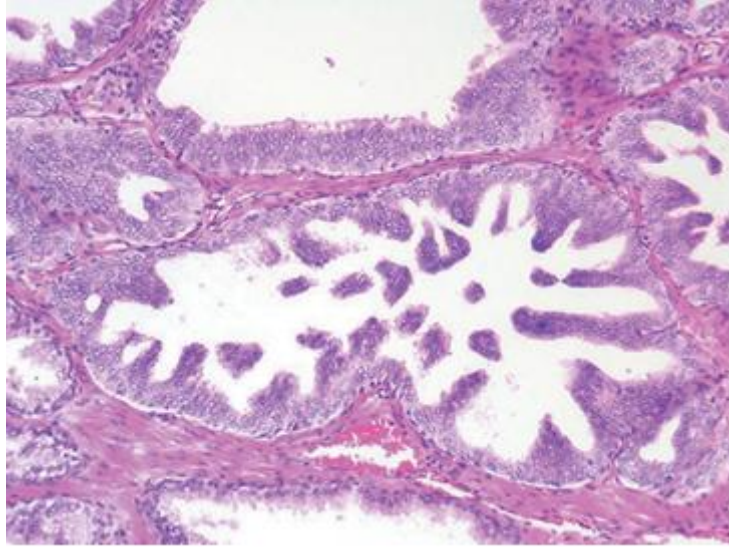
African-American men, who are at higher risk of prostate cancer mortality, appear to have a greater extent of PIN at any given age. PIN and prostate cancer lesions share a number of somatic genome abnormalities, including loss of DNA sequences at 8p and increased GSTP1 CpG island DNA methylation, among others.

Finally, transgenic mouse strains prone to developing prostate cancers typically develop PIN lesions in advance of the appearance of invasive cancer. PIN lesions are always asymptomatic and cannot currently be diagnosed or detected by any reliable means other than examination of prostate tissue histologically. In autopsy studies, the incidence and extent of PIN increases with age, as does the incidence of prostate cancer.

Notwithstanding the correlation, there does not seem to be causality. In addition, the authors do indicate that HGPIN can be reduced but they seem to fail to speak to the issue of total remission without any treatment. The question is therefore, is PIN a precursor of PCa? If it is or is not, is PIN the result of a genetic change as has been postulated by many? It would seem clear that the existence of remission of PIN would imply that it is not at all necessarily a precursor and furthermore that it is not necessarily a genetic change for all PIN. That is can there be a morphological PIN that is genetic and not remissionable and one which is remissionable. Remissionable implies the existence of apoptosis that is a natural cell death or perhaps a cell death due to some immune response.

4.3.2 PIN Morphology

Prostatic Intraepithelial Neoplasia, PIN, is a growth within the normal glands of more cells than should normally be there. The slide below depicts high grade PIN, HGPIN. Note the PIN in the center shows significant cell growth in the existing gland as compared to the gland at the bottom which shows normal thinner growth.



The PIN shows papillae which are shooting out within the gland and there is also significant basophilic staining of the papilla cells whereas the normal gland has limited staining of the luminal cells. The key question is one of whether PIN is a precursor to PCa. Many articles state that it is but when one looks at the data there is still a significant area of doubt.

4.3.3 Some HGPIN Models

There has been an extensive amount of work in trying to create HGPIN from normal prostate cells. There are questions as to what cells the HGPIN derives from, for example basal or luminal, and then there are questions as to what genetic changes result in PIN. As with so many parts of the puzzle there are no single set of answers. We start with the recent Goldstein model and use it as a basis. Then we look at other models and specific genes expressed. We defer until later the issue of pathways.

1.1.1.3 The Goldstein Model

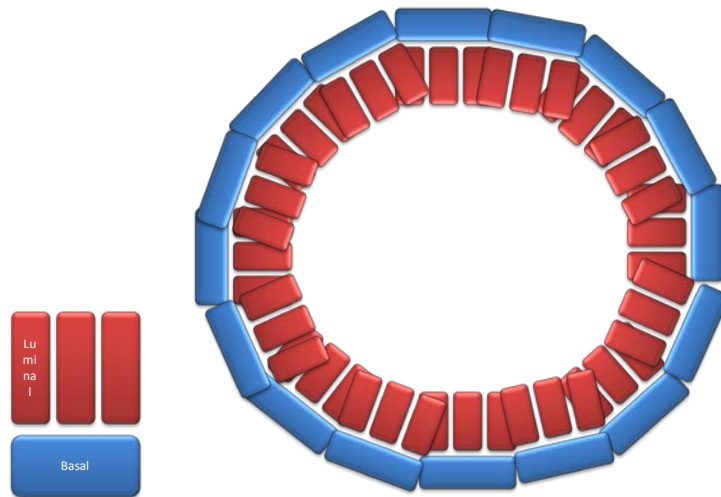
A novel set of experiments on prostate cancer were based on the work by Goldstein et al at UCLA. Understanding this work is useful in understanding both HGPIN and PCa. Goldstein et al demonstrate that one set of elements in the intracellular pathways if disturbed in a certain manner can result in morphological changes that first become HGPIN and then mode to PCa. The essential usefulness of this work is that it allows for a demonstrable relationship first between genetic change and histological change and second that changes in pathway elements lead to progression.

Simply what they did was to take two types of prostate cells, the basal and the luminal, tag them with surface tags, inject them into a mouse, and saw that only the basal cells grew, then they added two genes encoding for putative cancer pathways, and they saw that the basal cells grew to basal and luminal, like PIN, and then finally they added an AR, androgen receptor gene, and voila, prostate cancer. Result, showing how a specific pathway can generate cancer.

Let us go back and look at this a bit more.

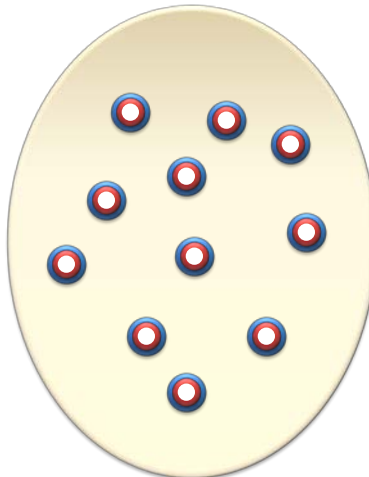
1. First the prostate has cell collections which act as glands with basal cells at the base and luminal cells on top. The luminal cells secret to the gland, the luminal space. This we show below.

Normal Prostate Gland

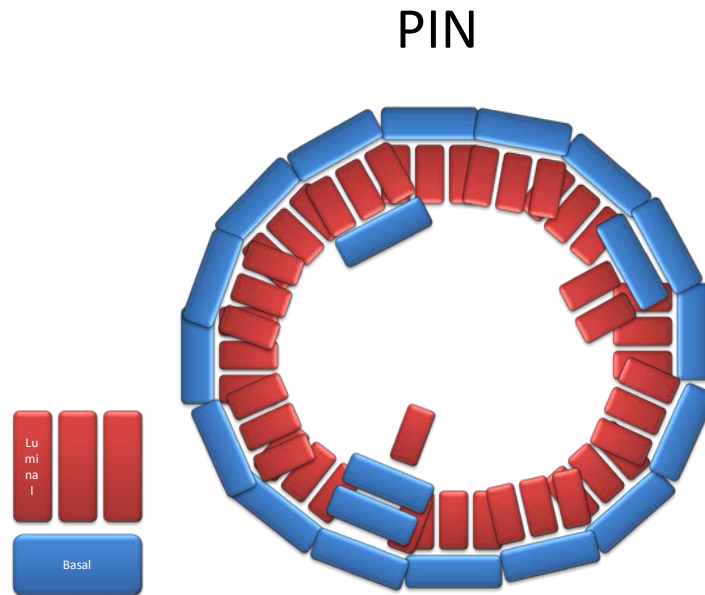


2. The normal prostate looks like what we show below, about 35-50 of these glands, and then surrounding material of muscle, blood supply, nerves, and lymphatics. The glands stand apart and they secrete fluids into the lumen, the open parts of the gland. In between is the stroma composed of nerves, blood vessels and other connective tissues.

Normal Prostate

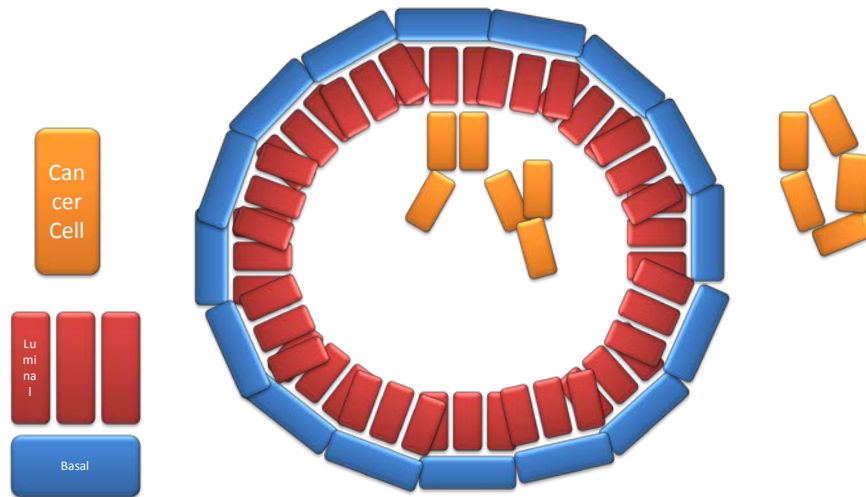


3. Now sometimes we see PIN, prostatic intraepithelial neoplasia, which is a growth of normal cells but not where they are to be. We may see the basal cells growing outwards and even some more luminal cells as well. The sign may be an increase in PSA since we have more luminal cells but the percent free PSA may stay high since the luminal cells are health ones. We show this below:

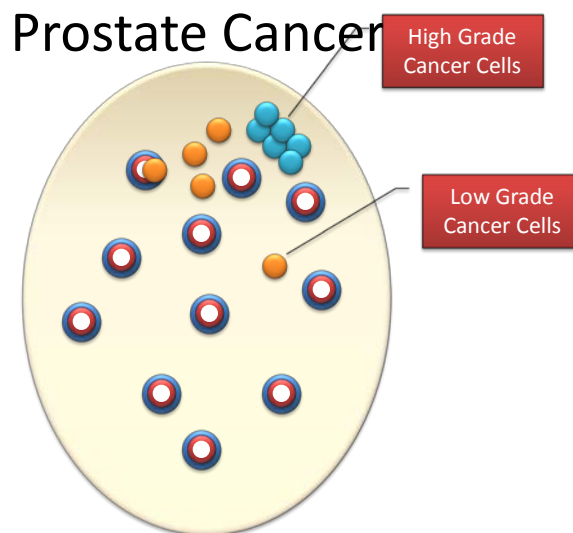


4. Then we may get prostate cancer, PCa, where the luminal cells types start to appear and grow without bound. The question is, where did these cells come from, other luminal cells or basal cells, or what. This is the question that the authors addressed with this elegant experiment. There is also the key question of whether it is just one cell that starts it or if the changed basal cells grow and if the environment switches many on over time. The latter effect is similar to that which has been observed in melanoma. Below we show what happens next,

Prostate Carcinoma

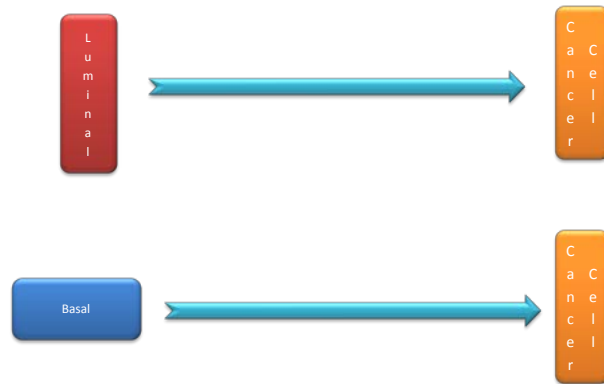


Looking at the prostate as a whole we then may see what appears below. Namely we may see low grade cancer cells and then clusters of high grade cancer cells, this leads to the Gleason grading system.



5. Thus the question posed by the authors was the one which asks from what cell does cancer begin? Their answer suggests the basal cell.

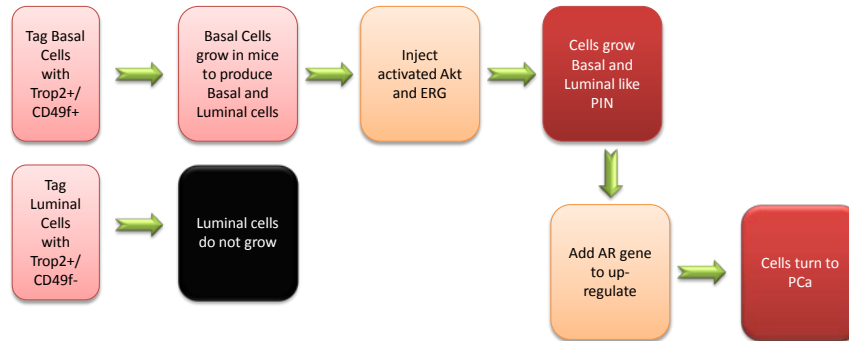
The Transition?



6. Pathways have been studied for PCa extensively and we shall discuss them in some detail.

But the authors took a simple approach and looked at three genes in the putative pathway process. This is shown below:

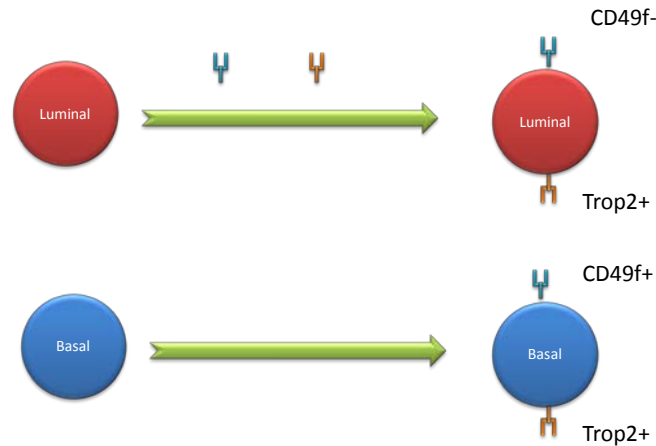
Goldstein Process



First they showed that only basal cell proliferate into both basal and luminal. Then they added ERG and Akt genes known as key in the pathways, and they obtained PIN, and then they added AR, the androgen receptor to drive the previous two genes and the result was PCa.

They were able to keep track of basal and luminal cells by tagging them with cell surface markers, as shown below. Basal was positive for both and luminal positive for one and negative for another, a good example of tracking the cells as the transform.

Tagging



As to the two initial genes we have:

(i) Akt: There are in humans three genes in the "Akt family": Akt1, Akt2, and Akt3. These genes code for enzymes that are members of the serine/threonine-specific protein kinase family. Akt1 is involved in cellular survival pathways, by inhibiting apoptotic processes. Akt1 is also able to induce protein synthesis pathways, and is therefore a key signaling protein in the cellular pathways that lead to skeletal muscle hypertrophy, and general tissue growth. Since it can block apoptosis, and thereby promote cell survival, Akt1 has been implicated as a major factor in many types of cancer.

(ii) ERK: Extracellular signal regulated kinases, ERK, are protein kinase signaling molecules involved in the regulation of meiosis, mitosis, and postmitotic functions in cells.

This study still leaves several open questions:

1. Is the clonal theory of cancer still standing or can a single cell transform and then induce other cells via chemical signaling.
2. Is the basal cell the only one. There appears to be some issues here and the review article looks at these.
3. Is PIN an artifact or a precursor. Clinically men with PIN have a slightly higher risk of PCa but not a substantially higher as would be argued in this model. In fact men with PCa do not always have PIN and men with PIN do not always get PCa.
4. Is this just an artifact pathway, the true pathway, one of many pathways.

5. If we can duplicate pathways can we then better control the disease.

6. What does this tell us about detection and staging.

1.1.1.4 Other Models

The Goldstein et al model is but one of several which have taken this approach. There are others and the results are not always consistent. Two of them are discussed as follows:

1. Yen et al (2003) have reported on a murine model which demonstrated that by implanting c-Myc genes into a mouse that it resulted in murine PIN and then shortly thereafter PCa. Yen et al also shown loss of NKX3.1, a tumor suppressor gene, which is putatively involved in PCa as well as PIN. NKX3.1 is a 8p21 gene whose function is to generate the Homeobox protein²⁵. It is known to be suppressed in familiar prostate cancer and in the case of Yen it is reduced in its expression as well.
2. Lawton and Witte discuss the generation of PIN by means of lentivirus infection via an siRNA which is a knock out for PTEN.

4.3.4 HGPIN, A Precursor of PCa?

There has been an extensive amount of literature claiming that high grade prostatic intraepithelial neoplasia, HGPIN, is a precursor to prostate cancer, PCa. The research has gone as far as delineating genetic changes which ultimately lead to metastatic PCa. However, at the same time it is not uncommon for HGPIN to regress and totally disappear. This would seem to counter the theory of genetic change and resulting morphological change of the prostate acini cells.

Moreover there have been many murine models of HGPIN which have been induced with specific genetic changes in specific pathways which lead inexorably to PIN and then to PCa. Likewise there have been many microarray analyses of HGPIN demonstrating the presence or absence, enhancement or deactivation, of the same or similar genes. Yet again there is at time spontaneous remission.

Thus it begs the question; what causes the remission of HGPIN? Is it possibly akin to the remission seen in certain cancers, a remission generated by an immune response effect, as discussed by Rosenberg? Or is it a pathway apoptosis that occurs as a natural course of having aberrant genes?

1.1.1.5 Key Questions

Let us begin with what we assume is known:

²⁵ Pecorino, Cancer, p 114.

1. HGPIN is driven by underlying progressive and non-changeable changes in the genetic structure of benign cells in the prostate glands.
2. There is a putative association between HGPIN and PCa, reflected in an increased incidence of PCa when HGPIN is present.
3. PCa like most other cancers is characterized by the clonal model, namely one cell becomes aberrant and all other cancers cells are daughter cells of the aberrant clone.
4. PCa is known to result via a set of genetic changes resulting in the cell growth outside of the gland and the creation of malignant glandular structures wherein additional genetic changes occur and result in a less structured morphology and then metastasis.
5. HGPIN regression is seen. This means that the HGPIN cells totally disappear resulting in a purely benign appearance of the prostate glands. It begs the question of; do they cells die or are they attacked and destroyed or is there some reversion mechanism? PIN is a proliferation, so any continuation of cell existence would imply at best a morphological change of say the nucleus and nucleoli but not the total cell count, namely the clustering of many cells in the gland. Thus in regression we do not know what happens or how.

Thus these observations pose the following questions:

1. What causes the disappearance of multiple clusters of HGPIN? Is it apoptosis of some form, an immune response, a genetic switch, or something else?
2. Has there been any extensive studies of HGPIN regression to understand how it arises?
3. If HGPIN regression is based upon some to-be-understood mechanism, can that same mechanism be applied in some form to PCa?
4. Does HGPIN, which is regressionable, have certain cell surface markers which are presentable to the immune system and thus enable enhanced immune responses?
5. Is there a stem cell created when PCa evolves and is PIN lacking in such a stem cell?

The literature demonstrates how to create PIN. There are a few presentations on how to regress PIN²⁶. However the nexus of forward PIN progression and backward PIN regression is not complete. We attempt herein to review this in some detail and then to place it in a structure for further analysis and study.

As a natural extension to these questions we can then ask similar ones regarding PCa. How does PCa progress and what are the pathway dynamics related to that progression.

²⁶ Narayanan et al using NSAID.

1.1.1.6 An Example

Let us begin with a simple example. A 68 year old male is examined due to an increase in PSA from 1.5 to 2.3 in a one year period. The DRE is normal but there is a family history of a first degree relative who died from an aggressive PCa, at 79 years of age. Re-measuring the PSA from two independent sources yields values of 1.8 and 1.9 two months after the raised PSA.

A 20 core biopsy is performed and the results are as follows:

- A. Prostate, right apex, biopsy: Benign prostatic glands and stroma.*
- B. Prostate, left apex, biopsy: Prostatic intraepithelial neoplasia, high grade, focal. Glandular hyperplasia of prostate.*
- C. Prostate left peripheral zone, biopsy: Prostatic intraepithelial neoplasia, high grade, focal, Glandular hyperplasia of prostate.*
- D. Prostate, right peripheral zone, biopsy: Benign prostatic glands and stroma.*
- E. Prostate, transition zone, biopsy: Prostatic intraepithelial neoplasia, high grade, focal Glandular hyperplasia of prostate.*

After an eight month period PSA was measured again and this time it was 2.0. A second biopsy was performed using 24 cores. The results are:

- A. Prostate, right apex, needle core biopsy: Benign prostatic tissue with very focal and mild acute inflammation.*
- B. Prostate, left apex, needle core biopsy: Benign prostatic tissue.*
- C. Prostate, right mid, needle core biopsy: Benign prostatic tissue.*
- D. Prostate, left mid, needle core biopsy: Benign prostatic tissue.*
- E. Prostate, right base, needle core biopsy: Benign prostatic tissue.*
- F. Prostate, left base, needle core biopsy: Benign prostatic tissue.*
- G. Prostate, transition zone, needle core biopsy: Benign prostatic tissue.*

This is a clear case of total HGPIN regression. The question then is, how common is this and what is its cause, and if regression can be obtained how it might be achieved clinically?

4.4 PCA HISTOLOGY AND GRADING

In this section we provide more detail on grading of PCa. The emphasis here is upon histological change and does not reflect any changes in specific gene pathways.

Prostate Cancer is simply the growth of abnormal glandular like structures outside of the normal prostate glands the resulting continued growth of the cells making up those structures both within and without the prostate. The PCa cells take over the stroma, pushing aside the normal stromal cells and then migrate in a metastatic fashion throughout the body.

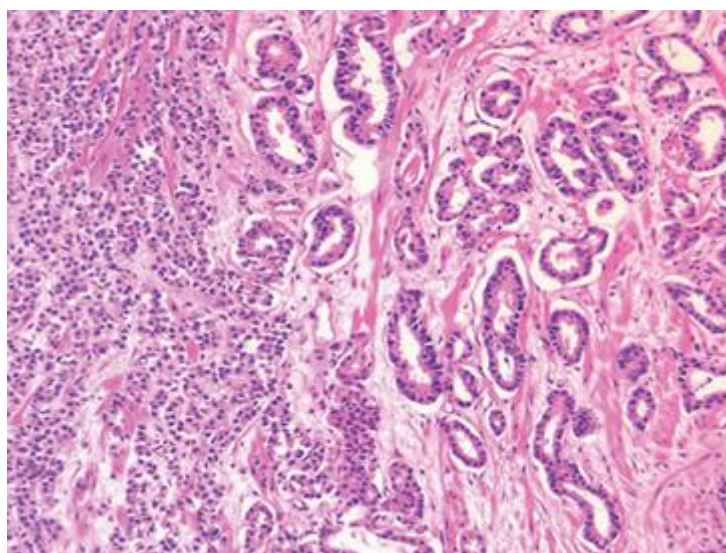
We will use the Gleason grading score as a means to characterize the level of cancer progression within the prostate.

4.4.1 Grading

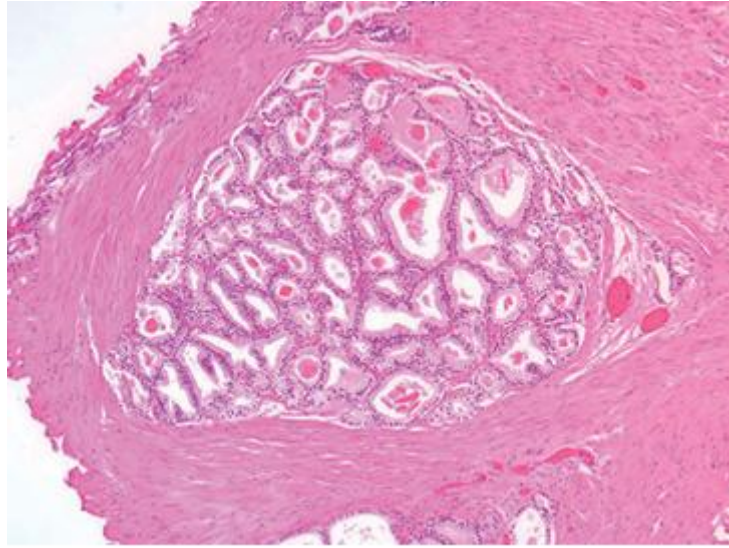
We present the grading system developed by Gleason. On the one hand this has been used as a gold standard for ascertaining future progress and yet it is still just a morphological tool. It fails to determine the pathways and regulators in a cell by cell basis.

1.1.1.7 Gleason 1

The following is a Gleason 1 grade tumor. Note that there are a proliferation of small glandular like clusters with dark basophilic stains and they are separate and have clear luminal areas. Gleason 1 is generally composed of many single and separate and closely packed glands of well circumscribed uniform glands. One rarely sees Gleason 1 grade tumors, and they are often found as incidental findings when examining for other issues.



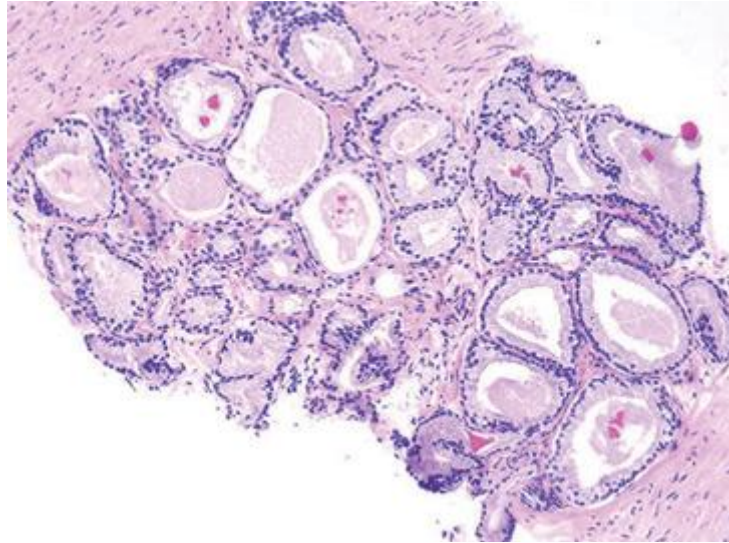
We show another view of a Gleason 1 below. This is especially descriptive of such a form because it appears almost as a single and isolated structure. The interesting question will be if this is PCa then if PCa is clonal is this cluster an aberrant outgrowth of a normal cells, if so which one, and if so is this just one cell growing. It appears that at this stage the intercellular signaling is still trying to function. However the clarity of cell form is being degraded.



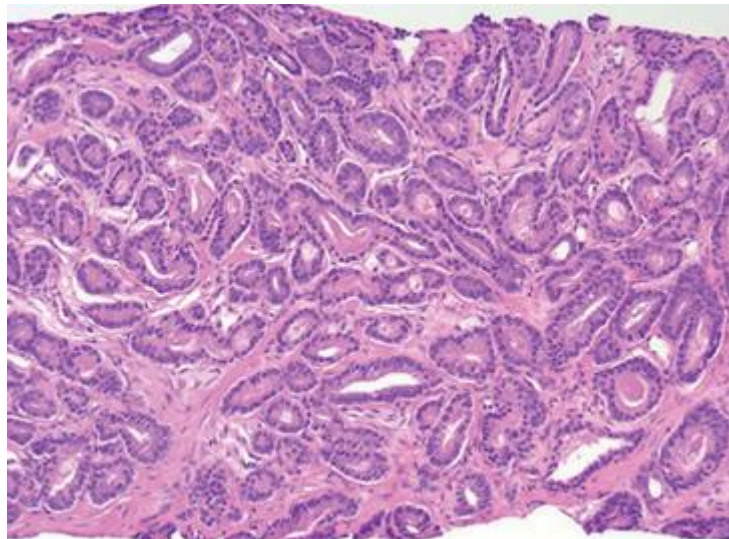
1.1.1.8 Gleason 2 and 3

Gleason 2 shows many more new glandular like cells but now of varying larger sizes. As Epstein notes: "*Grade 2 ... is still fairly circumscribed, at the edge of the tumor nodule there can be minimal extension by neoplastic glands into the surrounding non-neoplastic prostate. The glands are more loosely arranged and not as uniform as Gleason 1.*" We see those in the figure below which combines Gleason 2 and 3.

Gleason 3 is often composed of single glands. The Gleason 3 infiltrates in and amongst the non-neoplastic glands. Gleason 3 still can be seen as a separate gland and there are no single cells starting to proliferate. In Gleason 3 we still have some semblance of intercellular communications and coordination, albeit with uncontrolled intracellular growth. Again in the figure below we see both the smaller 2 and the larger 3 with gland structure being preserved and no separate cells proliferating.

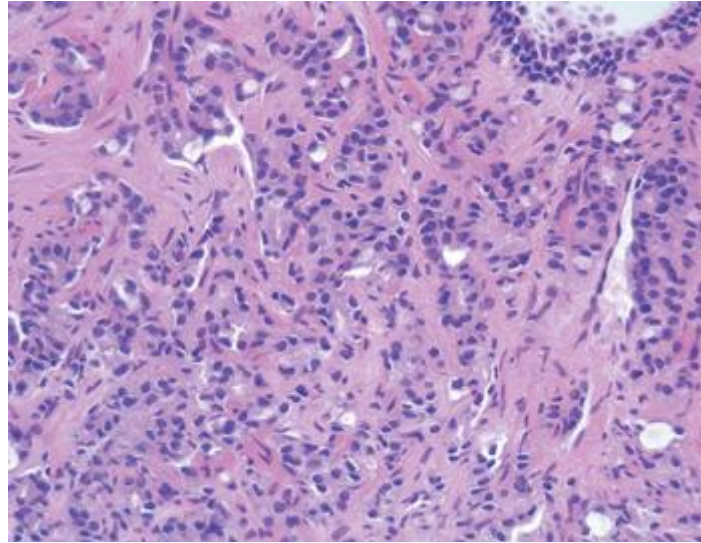


A Gleason 3 throughout is shown below.

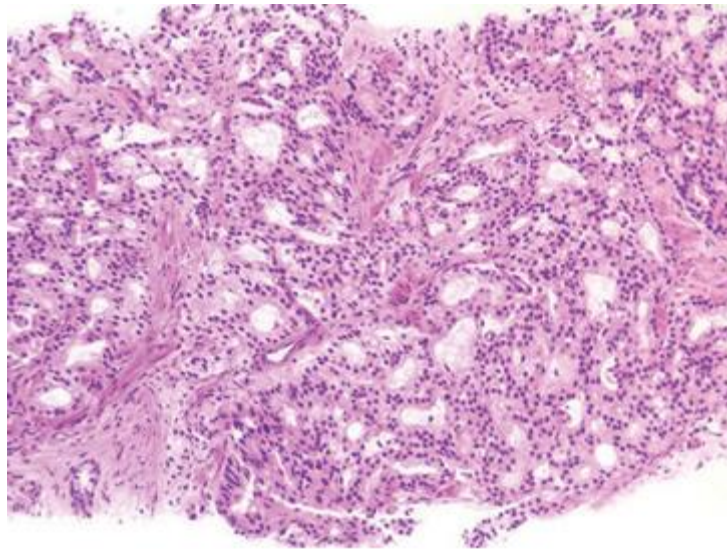


1.1.1.9 Gleason 4

Gleason 4 consists mostly of cribriform cells (perforated like a sieve) or fused and ill-defined glands with poorly formed glandular lumina. The glands appear to start to "stick" together. A Gleason 4 with a Gleason 3 is shown below. Note the sieve like structure and the closing of the glands.

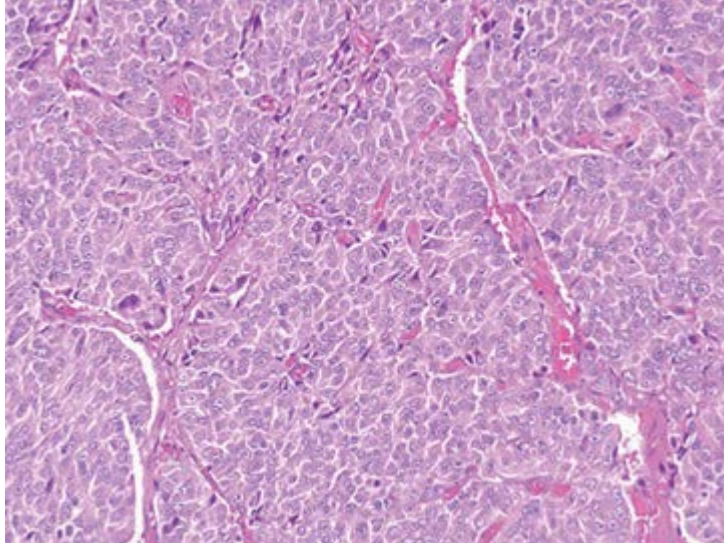


A Gleason all 4 is shown below. Note that the cells are sticking closed and the entire mass appears as a sieve like mass.



1.1.1.10 Gleason 5

Gleason 5 is a complete conversion to independent malignant cells. They have lost all intercellular coordination. As shown below it is a mass or mat or sheet of independent cancer cells and it has lost any of the sieve like structures. There may also appear to be some necrosis



4.4.2 Gleason Summary

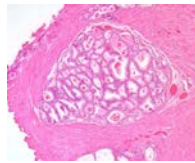
The Gleason scores are then determined by taking the predominant type and adding it to the secondary type. Thus a 4+3 yields a Gleason combined 7 but it is 4+3 and that is more aggressive than say a 3+4 with the same total score.

We repeat the grading commentary below.

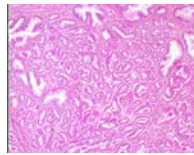
| Gleason 1 | Gleason 2 | Gleason 3 | Gleason 4 | Gleason 5 |
|---|--|--|---|---|
| Many acini with no basal layers and large nucleoli. Closely packed clumps of acini. | Many very small single separate glands (acini) with no basal layer and large nucleoli . Glands, acini, are more loosely arranged and not close packed. | Many small microglands extending throughout the stroma and out of the normal gland structure | Glands are now spread out and fused to one another throughout the stroma. | No gland structure seen, all luminal cells throughout the stroma with large nucleoli. |

The following chart is a summary of the progression.

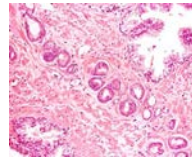
Gleason Grades



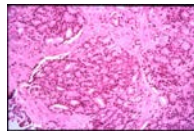
Gleason 1



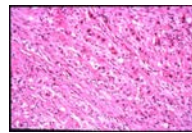
Gleason 2



Gleason 3



Gleason 4



Gleason 5

4.4.3 Models From Grading

In looking at the grading one may also hypothesize a possible path of progression. The steps appear to be:

1. Movement from existing benign gland to a separate but glandular like proliferation. Cells which would normally remain dormant go through a replication cycle, apoptosis and cell proliferation control seems lost. New glands appear clustered but appear separate.
2. Growth of the new glands makes them expand but remain morphologically glandular. They close packing begins to disappear and glands start to stand on their own. It is as if they are expanding and growing and the basal layer begins to disappear. Luminal like cancer cells start to be predominant.
3. Many small micro-glands start expanding and cell growth accelerates and the cells appear more cancer like but there is still some morphological glandular structure left.
4. The many glands have dramatically different shaped and start closing in one another and appear sieve like with small openings. They look as if they are losing any intercellular communications resulting in a common mat of cells.
5. Cells have lost any morphological form related to glands and appear as a mat of cancer cells replacing the stroma totally. No intercellular communications is left and cellular growth control has been eliminated totally.

These five steps are consistent with the Gleason grading but they also parallel the way the intracellular and intercellular controls are lost. We will look at these mechanism later.

4.5 REGRESSION

There has been some discussion of regression in the literature. We examine briefly three possible means here. However, there does not seem to have been any detailed clinical study or models, murine or otherwise, which have been used to ascertain the details which surround the regression issue. As we have seen above the current general understanding is that HGPIN is a clear and unambiguous predecessor of HGPIN, albeit regression is evident.

4.5.1 NSAID Regression

An interesting paper by Narayanan et al describes their work using NSAIDs as a means to reduce and in some cases eliminate PIN. They used specifically celecoxib and exisulind as the NSAID and they demonstrated that the use of these drugs did reduce PIN lesions. Now exactly why this happened one cannot determine. The authors present the factual results without any further interpretation. In addition there would not seem to be any rational explanation based upon the above overviews.

4.5.2 Androgen Deprivation Therapy Regression

In the paper by Kang et al they indicate that ADT, androgen deprivation therapy does reduce PIN²⁷. They state:

Our results demonstrate that ADT does cause PIN regression, and that there is heterogeneity in this effect with respect to hormonal duration. We propose for future prospective, multi-centered, randomized trials in which ADT impact on PIN is characterized further....However PIN response to ADT was not uniform as 16% of patients with ADT longer than 6 months had residual PIN, suggesting variable sensitivity of PIN to ADT.

Kang et al also noted in another paper:

Eighteen patients initially diagnosed with PIN who had no ADT were identified, and 28 with PIN who had ADT were also assessed. All patients who had had no ADT had residual PIN, whereas seven of 28 receiving ADT had no residual PIN ($P=0.043$). The evaluation of ADT between responders and nonresponders showed a statistically significant association between PIN regression and the duration of ADT ($P<0.001$).

However, the response of PIN to ADT was not uniform, as 16% of patients on ADT for >6 months had residual PIN, suggesting variable sensitivity of PIN to ADT.

²⁷ http://meeting.ascopubs.org/cgi/content/abstract/24/18_suppl/4648

4.5.3 mTOR Inhibition

The mTOR gene can be activated by the Akt gene which in turn can be activated by the suppression of the PTEN gene. This is but a small segment of a pathway. mTOR then

Thus there seems to be an ability to eliminate PIN via ADT. In this case there is some clear pathway dependence. mTOR is short for “mammalian target for rapamycin”²⁸. mTOR when positively enhanced by activation can result in cell growth by the up-regulation of protein synthesis. Akt regulates mTOR via the negative regulation of an intermediate pathway element the gene product TSC2 which inhibits mTOR.

By inhibiting TSC2 the inhibition of mTOR is reduced and in fact mTOR expression and actions are increased. It is this change which Majumder et al used to create PIN.

Majumder et al state that they were able to reverse PIN in murine models by managing mTOR pathways. The use of rapamycin was a reasonable approach for pathway control. Akt induced PIN was totally controlled by mTOR and reversal allowed regression of the PIN.

The above three are a few of the known mechanisms related to regression. There may be many others yet to be determined but the existence of these may assist in understanding the possible options.

4.6 SUMMARY

One can gain some insight into PCa and its evolution by understanding the histological changes. PCa starts out with a simple glandular structure, and then as a result of many changes begins to have within the gland excess growth, thus the PIN, and then the growth of new quasi glands, small and somewhat poorly shaped ones, which become the early signs of PCa. Then the differentiation of gland and stroma begins to disappear until the glandular elements are almost blocked from any possible view. The malignant cells have taken over the prostate and at this stage metastasis may very well have begun as well.

It will be useful to maintain a reasonably high level understanding of these cellular changes. They will be the driver of any model. We will now move on to understanding the genetic factors related to these changes.

²⁸ Bunz, pp 192-194.

5 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. All of this examination is to be focused on the cell cycle. This Chapter is a discussion of what is necessary to understand the importance of the cell cycle. The cycle is what often is broken in cancer cells, namely the cell reproduces again and again.

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 certain 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.

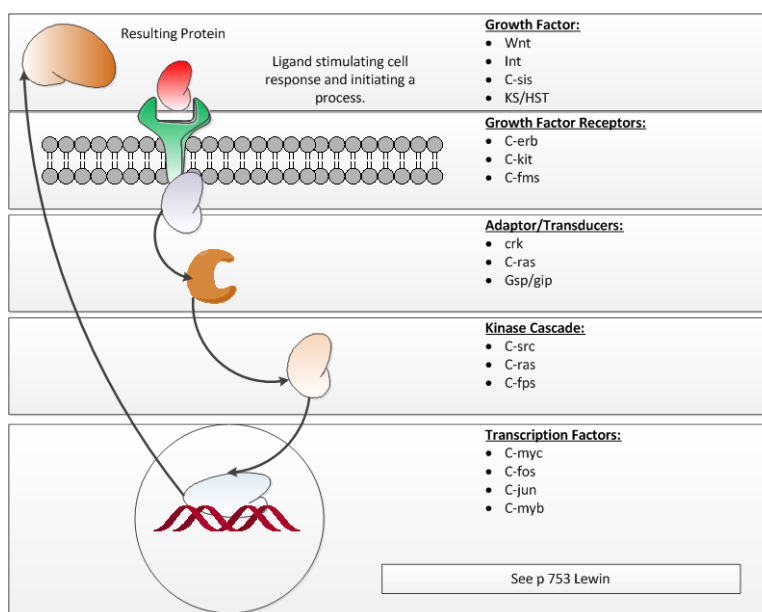
Note that this is a complex process.

| | | | | | |
|----------------------|--------------------------|------------------|----------------|--------------|-----------------|
| Ligand | PDGF | Insulin | Growth Hormone | IL-1 β | 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.

5.1 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:

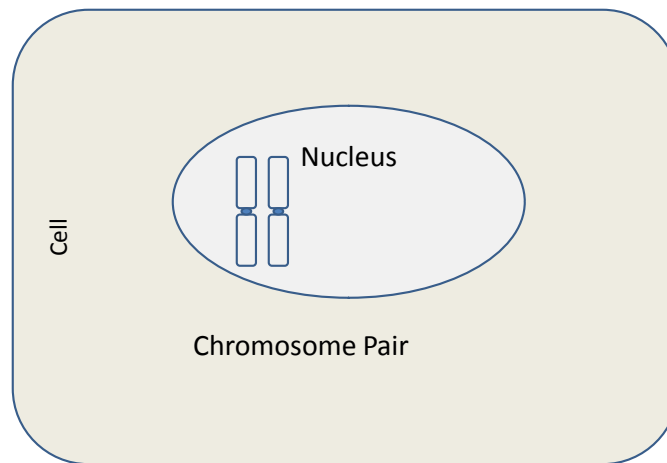
1. Gene structure and operation. This includes the basic Watson and Crick model as is currently understood. The development that we use is a functional model and note one that would be more familiar to the biologist. In all our analyses we will build models of functions and leave the basic principles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

5.1.1 Chromosomes and Genes

Let us 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.

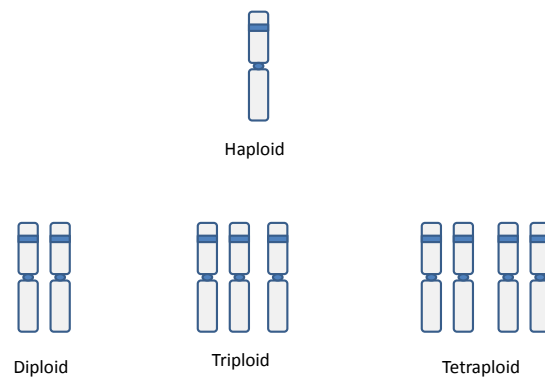
The Figure below is a graphic of a typical cell showing the nucleus and one of the chromosome pairs. This graphic is not at all what one would see in reality but it is typical of the generic elements.



5.1.4 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:

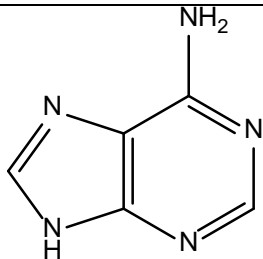
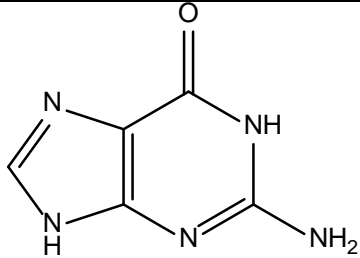
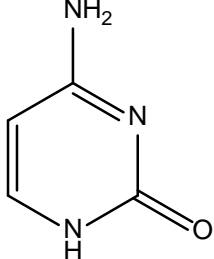
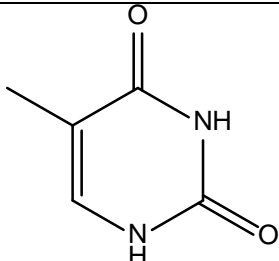
Haploid: The haploid is the single chromosome strand that one may be able to see in the sex cells. The haploid is a single stranded non-binded collection of DNA.

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

5.1.5 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.

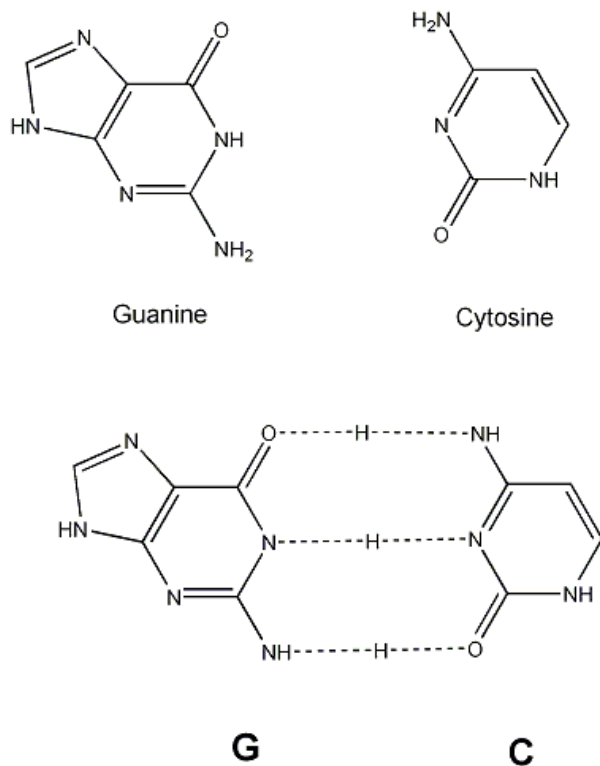


Figure 1 CT Base elements and their bonding.

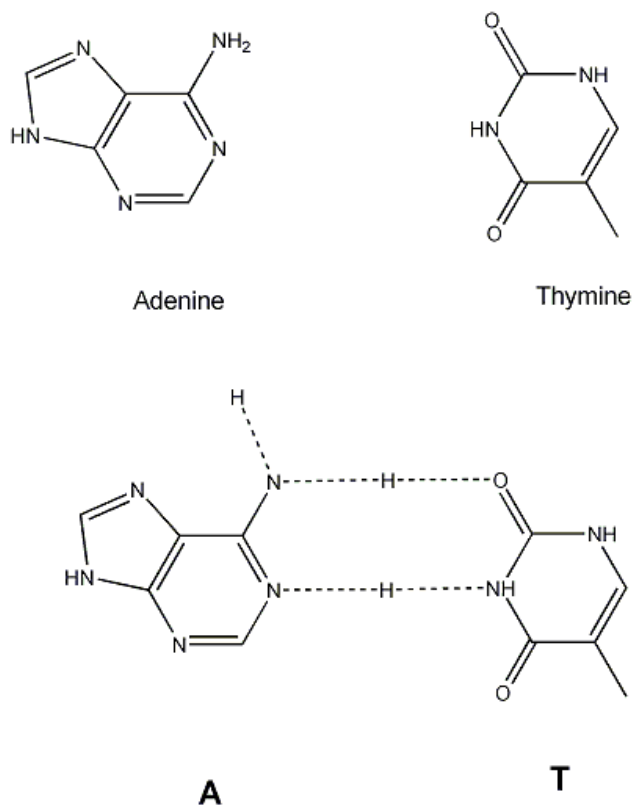
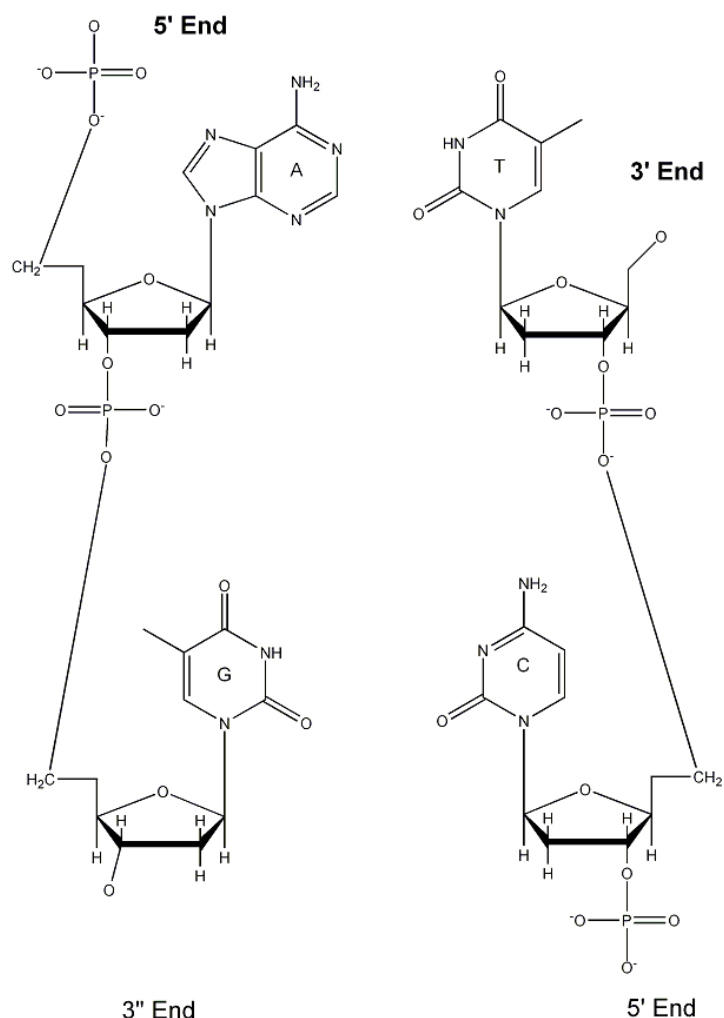


Figure 2 A-T Base Pairing

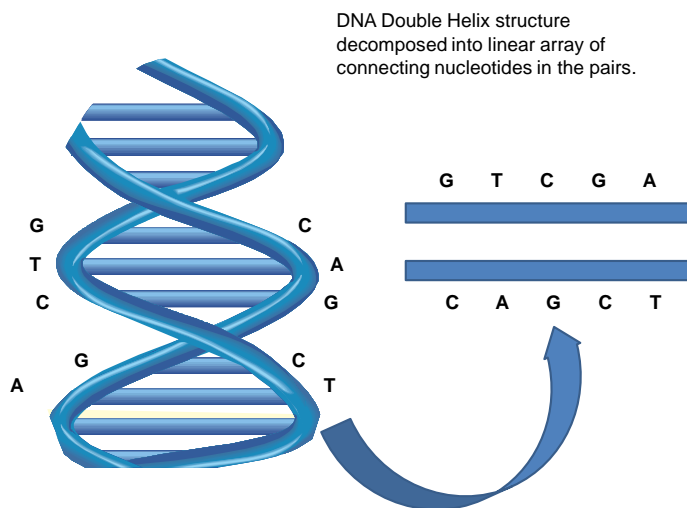
Now these Base Pairs are connected to sugar molecules, a cyclic ribose, to create a Nucleoside, such as deoxyadenosine. Then the nucleosides are enhanced with a phosphate constellation, a phosphorous molecules surrounded by oxygen and hydrogen. This combination of the nucleoside and the phosphate is 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.



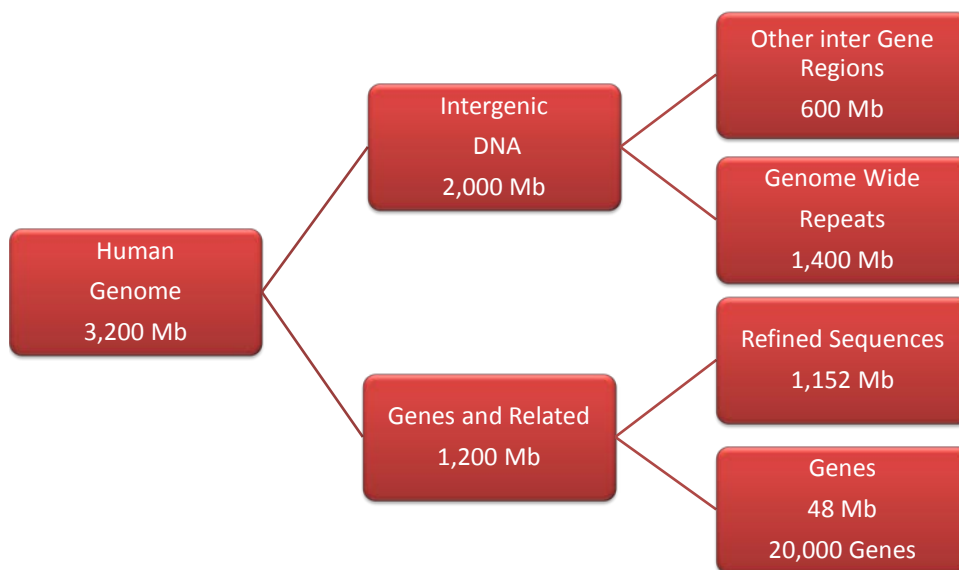
The nucleotides are then connected into the long DNA wrapped double helix which is generally well known. This is shown below. Our interest will be in the genes themselves and we will look at them in some detail. One of the key questions will be just what is a gene? That will be a challenging question. 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.



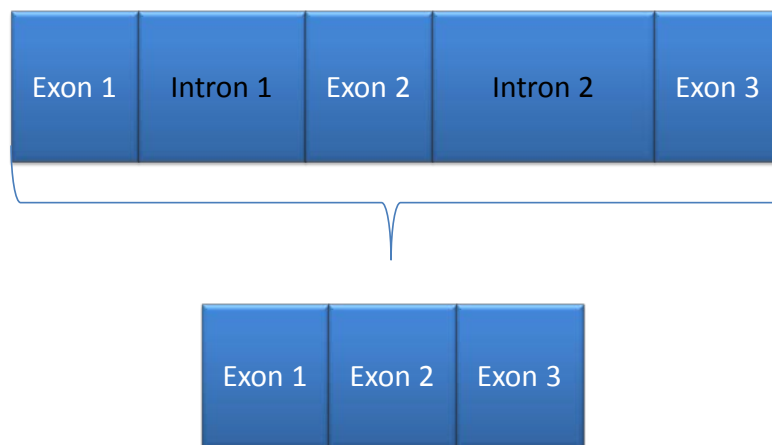
Ref: Watson et al, Molec. Bio Gene 5th Ed, p. 137

5.1.6 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 genes²⁹. 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.



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

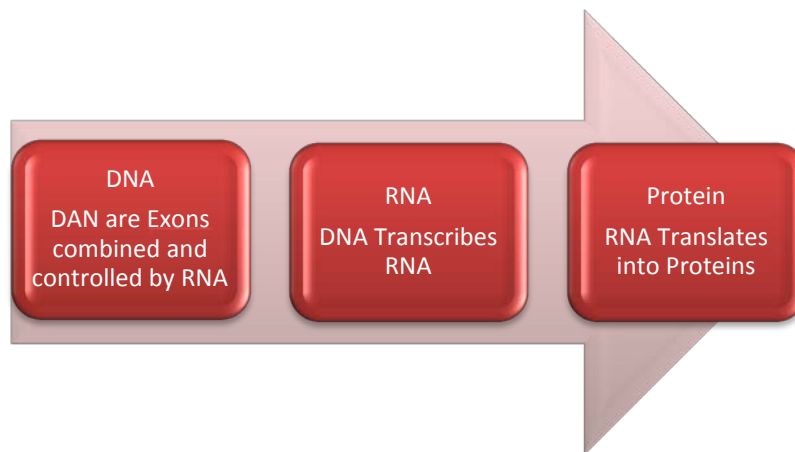
²⁹ See Pennisi, Working the (Gene Count) Numbers: Finally, a Firm Answer? SCIENCE Vol 316 25 May 2007

<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>

"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. Its 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 later.



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.

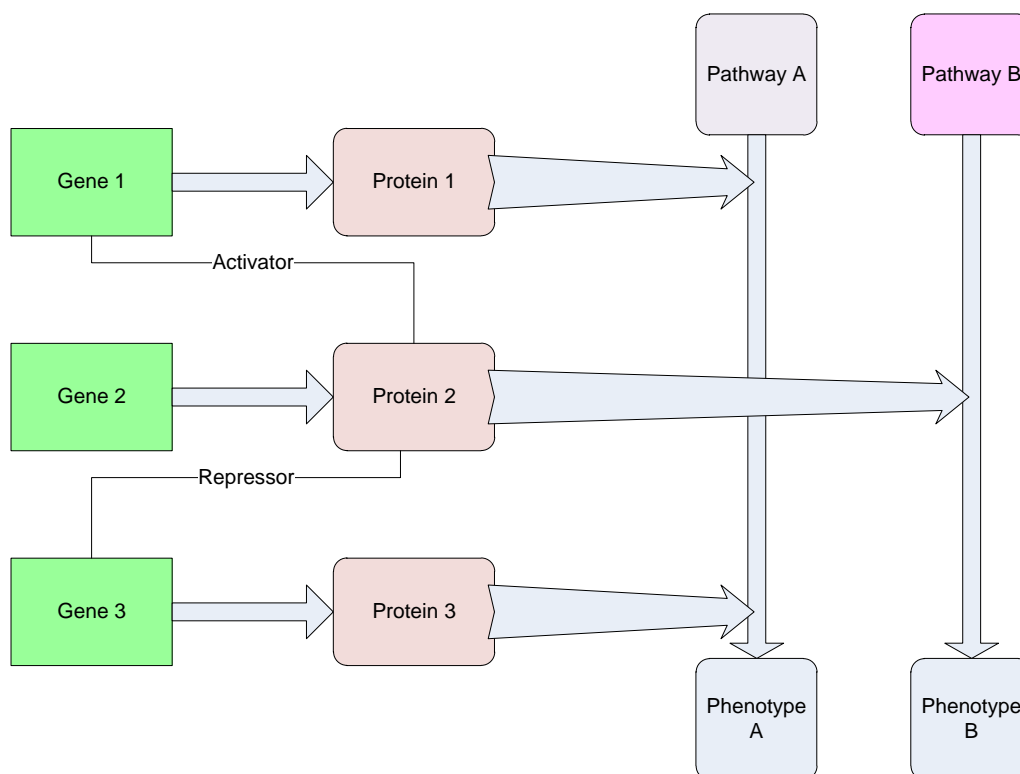
5.2 GENOTYPE AND PHENOTYPE

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.

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.

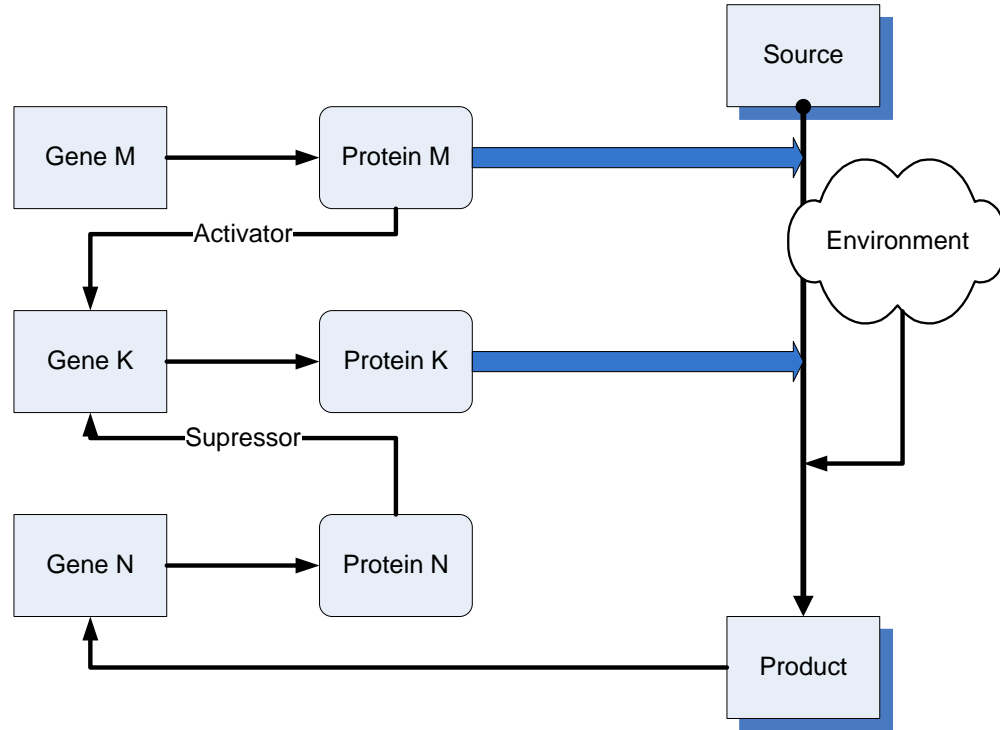


It is the output of this genetic process that we get the full temporal and spatial existence.

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

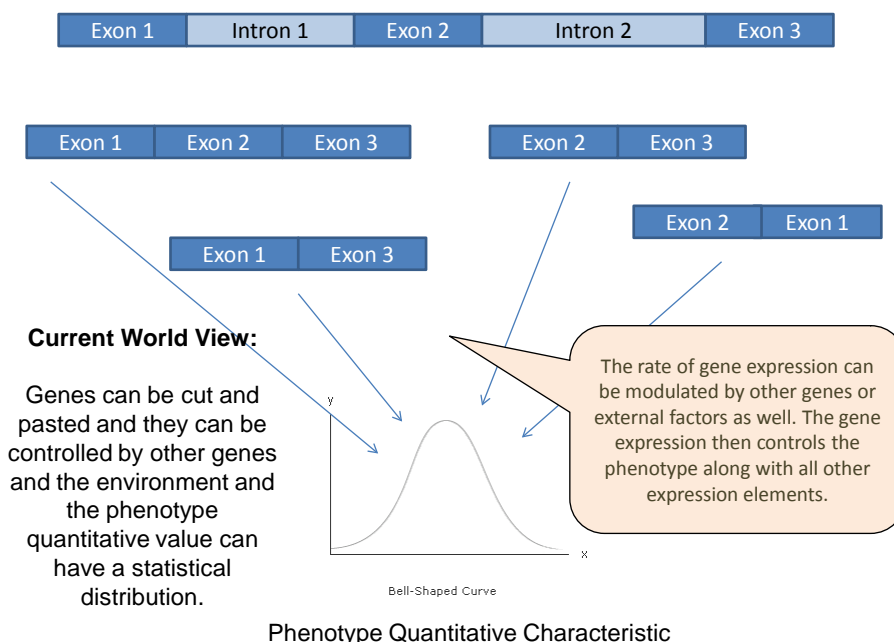
a complex process and exceeds what we would have imagined from the simple Mendelian gene theory.



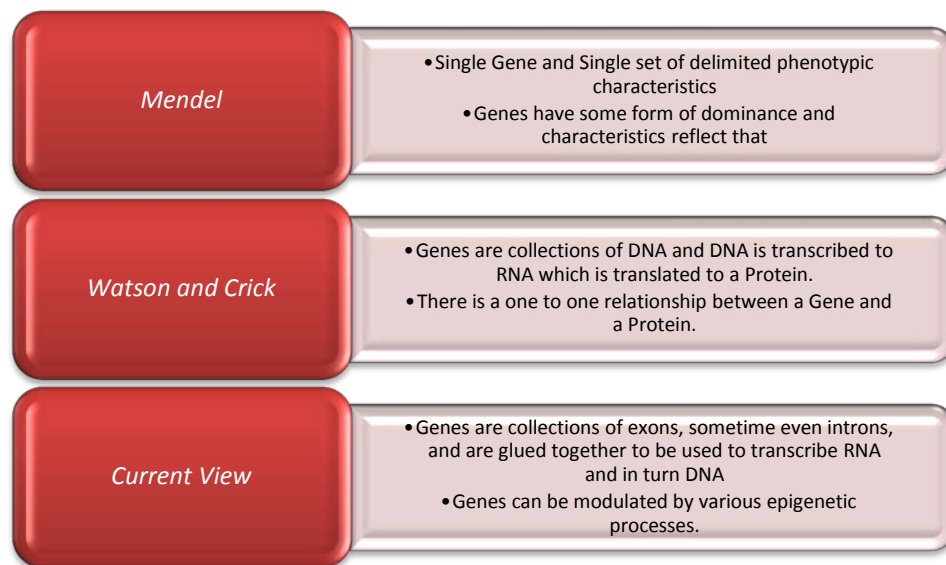
Now back one again to the Mendelian Gene model. Although Mendel and his model was not so rigidly simple, for he did admit some other influences as well as variation, we will call the simple Gene and Phenotype combination the Mendel Model. Namely in this model we assume the existence of a Gene and then 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.



The following graphic depicts the difference between the three models we generally consider. Mendelian models assume some construct of a gene. The Watson Crick model takes the gene a step further and relates gene to RNA to protein. In the current model there is substantial multilayer feedback complexity.



What is critical to understand in the current world view of genes and their effects is that there are many interlinking pathways. When we present the pathway models we will see that the present view is of pathways which have a linear non-feedback structure. However we know that they have substantial feedback. Feedback results in instabilities. Instabilities may very well be part of many malignancies.

5.3 GENETICS

In this section we present an overview of the classic gene expression analysis.³⁰ The Mendelian 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 Mendelian 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 later 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 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.

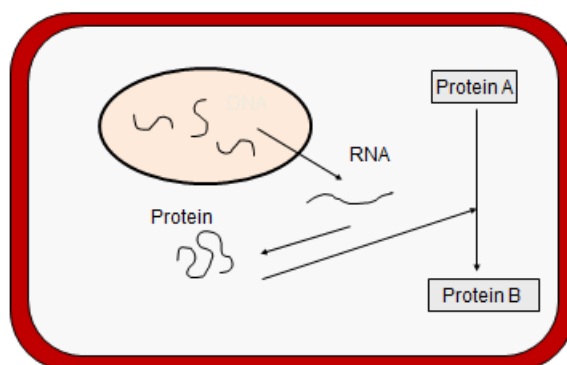
5.3.1 Cells and DNA

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.

³⁰ 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.

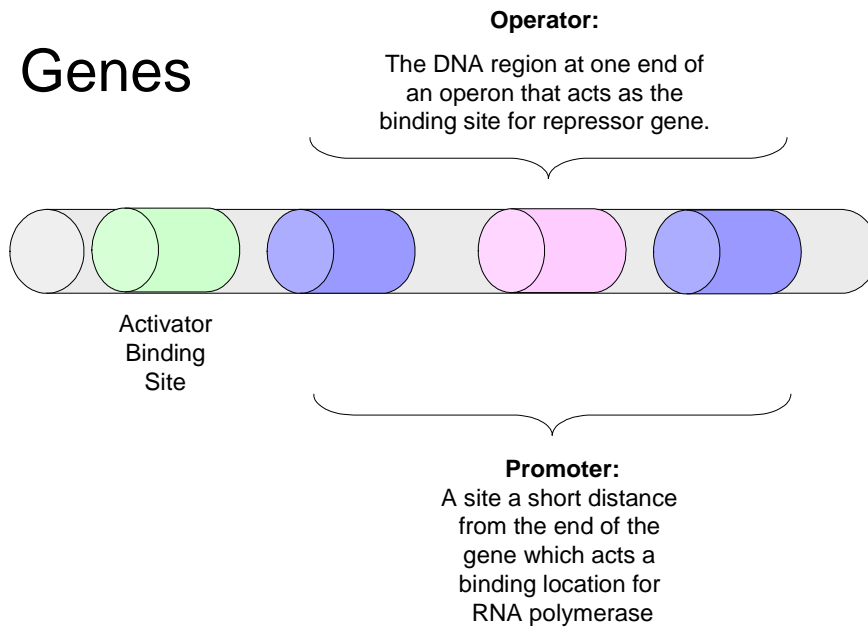
These two views have analogs in mathematical analysis; they are the time averages versus the ensemble average. In mathematical statistics we have the concept of looking at a single cell and time averaging say the concentration of a certain secondary. We know how it is produced and thus over some time window we can look at the average of say pelargonidin and we then 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 Wiener³¹. 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.

5.3.2 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 activator site which is where activator proteins can bind to start or enhance the expression of the

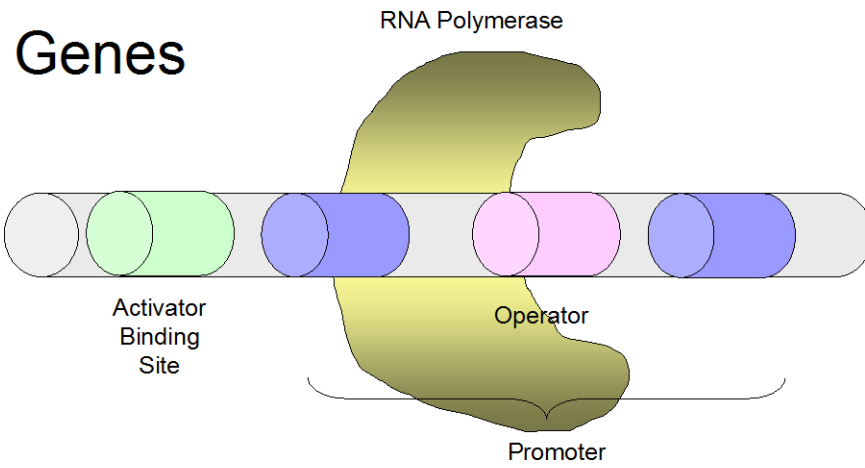
³¹ See McGarty, Stochastic Systems and State Estimation.

gene. The operator sits and the overall promoter sequence are shown down from the activator site.³²



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.

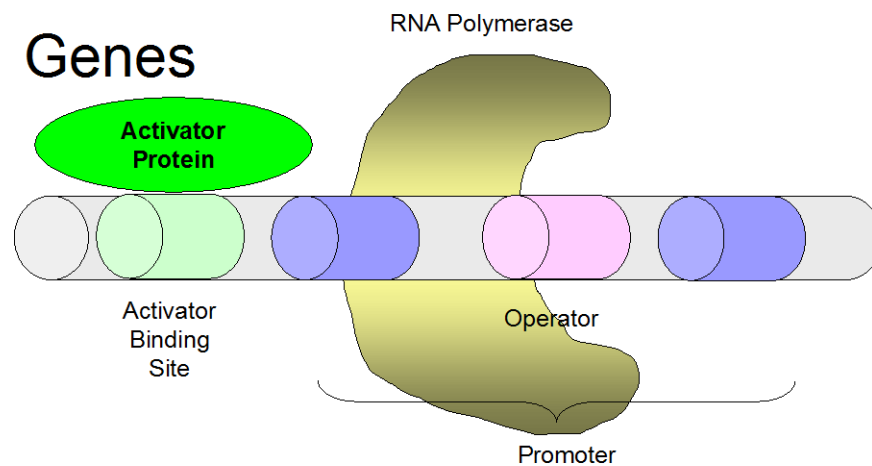
³² This is detailed in Watson et al. Also see Griffiths et al.



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

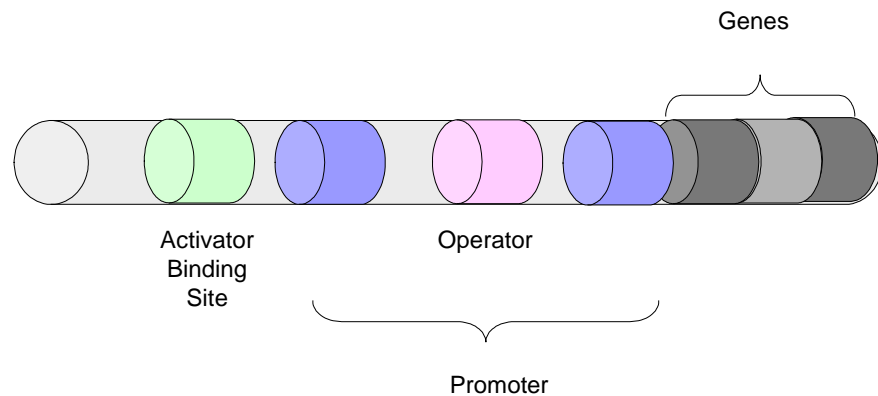
5.3.3 Activators

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.

Genes



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 = Output Protein Concentration

P_i = 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:

$$\frac{dP_o}{dt} = f(P_o(t), P_i(t), t)$$

$$P_o(0) = P_o^0$$

$$P_i(0) = P_i^0$$

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:

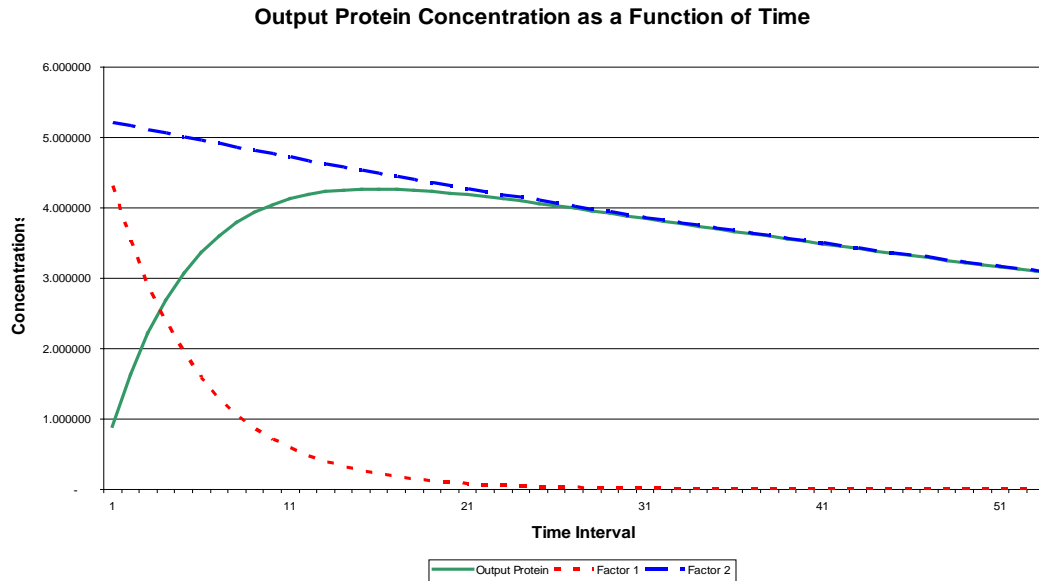
$$P_o(t) = k_{o,i}P_i(0) \left[\frac{\exp(-\lambda_i t) - \exp(-k_{o,o} t)}{\lambda_i - k_{o,o}} \right]$$

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.

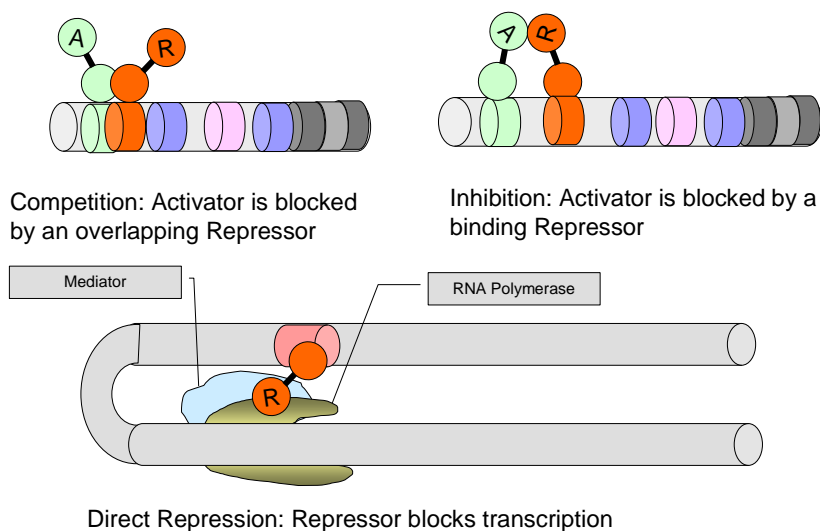


Note that the output protein concentration reaches a peak and then decays as per the driving protein. We will see this phenomenon again.

5.3.4 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.

Repressors



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.

5.4 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.

5.4.1 Approach: Engineering versus Science

The approach we take in this Chapter is an engineering approach rather than a biological approach.³³ 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.

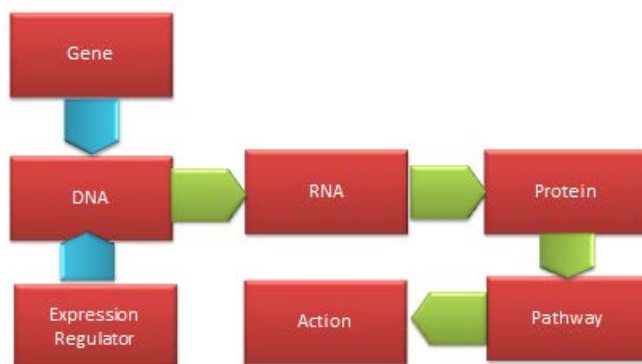
³³ There has been a significant set of development recently in analyzing genetic data from a systems perspective. In this Chapter we have taken such an approach. The recent work by such authors as Perkins et al, Vohradsky, Hatzimanikatis et al, and the recent book by Szallasi are seminal. However, there 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.

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 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.

5.4.2 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 hundreds 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.

5.5 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.

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.

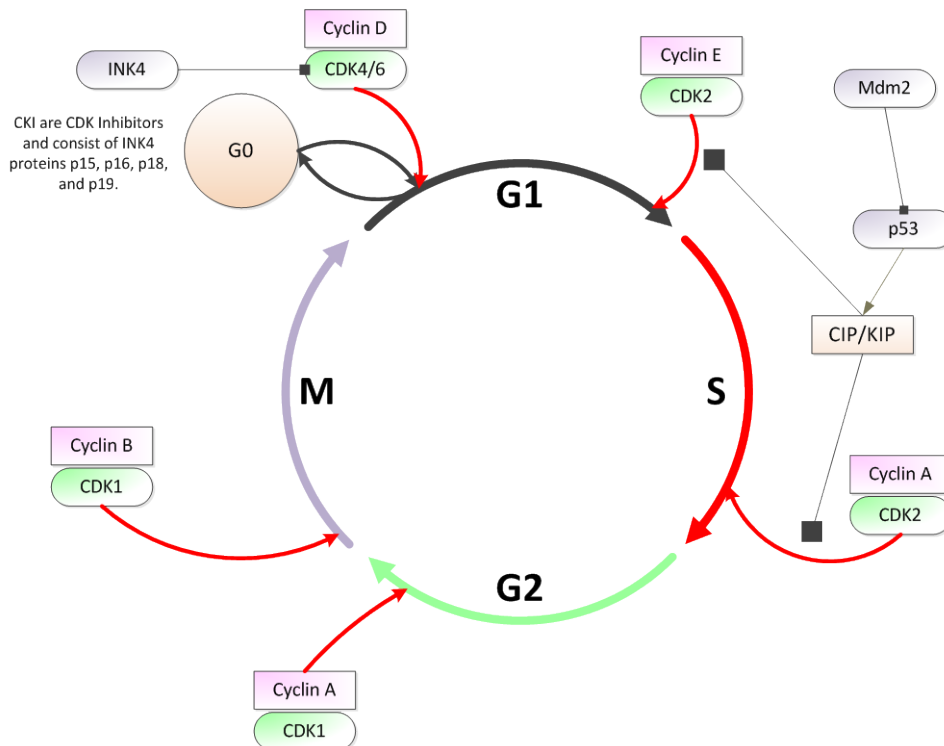
G1: Once the cell begins the G1 phase it is on its way to reproducing via mitosis.

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.

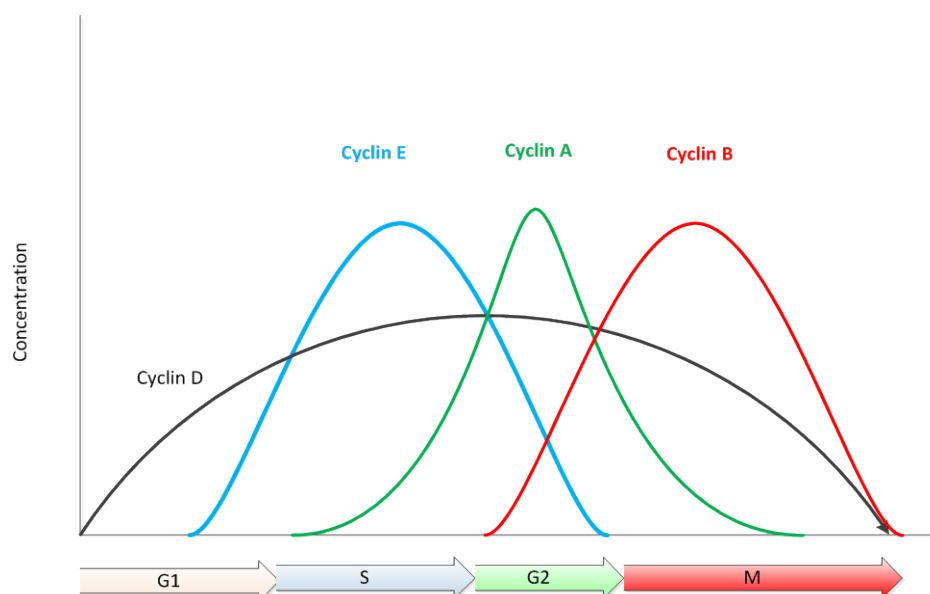
M: M phase includes mitosis and cytokinesis, namely the creation of two identical new cells.

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 are detailed in Morgan (Chapter 3).

| Protein ³⁴ | Gene | Function ³⁵ |
|--|-------------|---|
| 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. |

³⁴ <http://www.ncbi.nlm.nih.gov/gene/983>

³⁵ From <http://www.ncbi.nlm.nih.gov/gene/595> data bases as a source.

| <i>Protein</i> ³⁴ | <i>Gene</i> | <i>Function</i> ³⁵ |
|------------------------------|-------------|--|
| 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 phosphorylation 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, is observed frequently in a variety of tumors and may contribute to tumorigenesis. |

| <i>Protein</i> ³⁴ | <i>Gene</i> | <i>Function</i> ³⁵ |
|---------------------------------|-------------|---|
| Cyclin E (CCNE1) ³⁶ | 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:

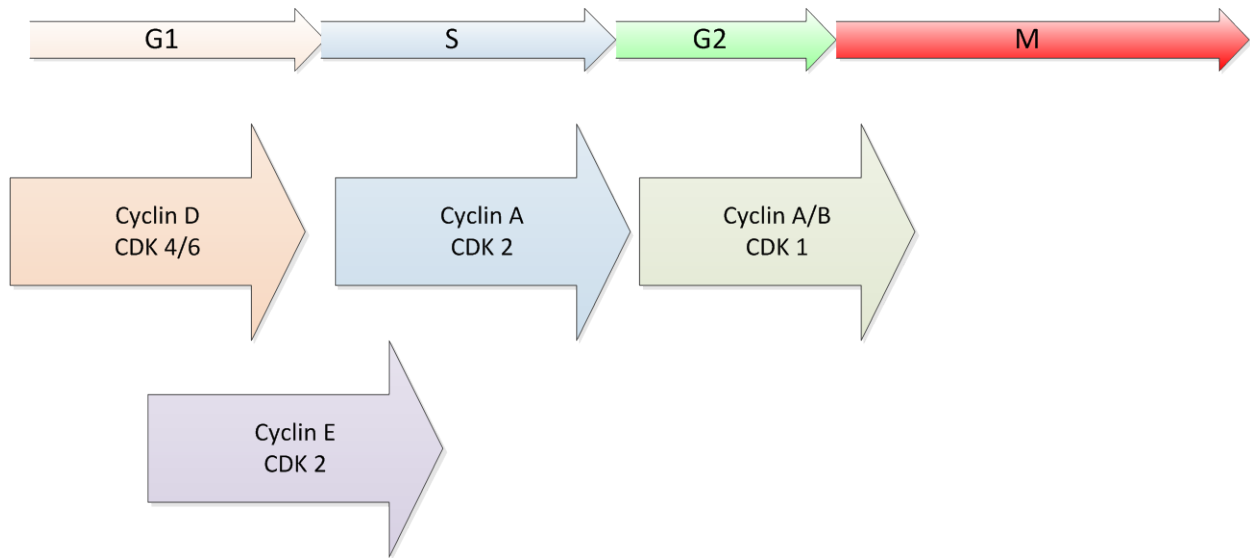
³⁶ <http://www.ncbi.nlm.nih.gov/gene/898>

| Protein ³⁷ | Gene | Function ³⁸ |
|--|-------------|---|
| 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 kinase 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. |

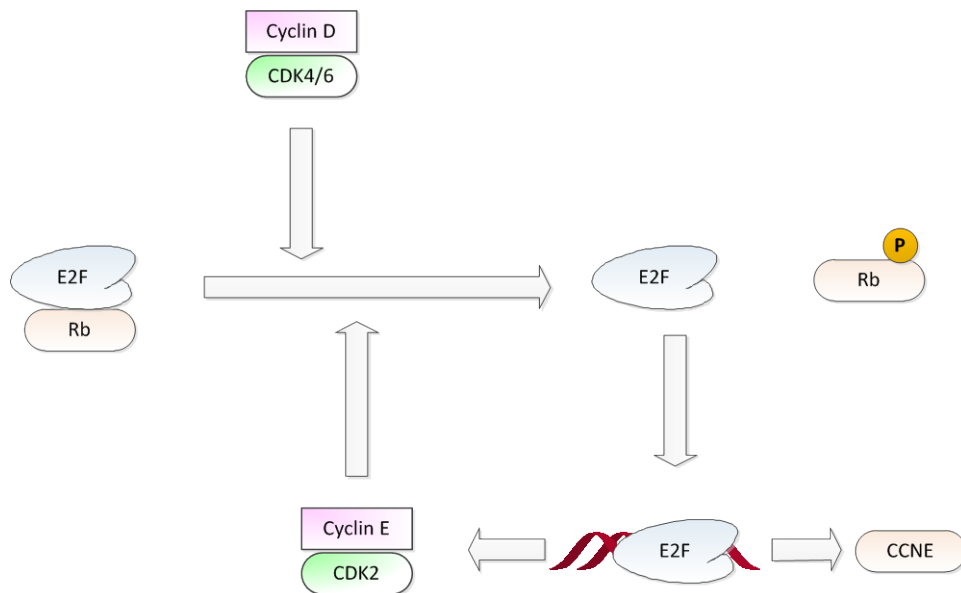
³⁷ <http://www.ncbi.nlm.nih.gov/gene/983>

³⁸ 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.

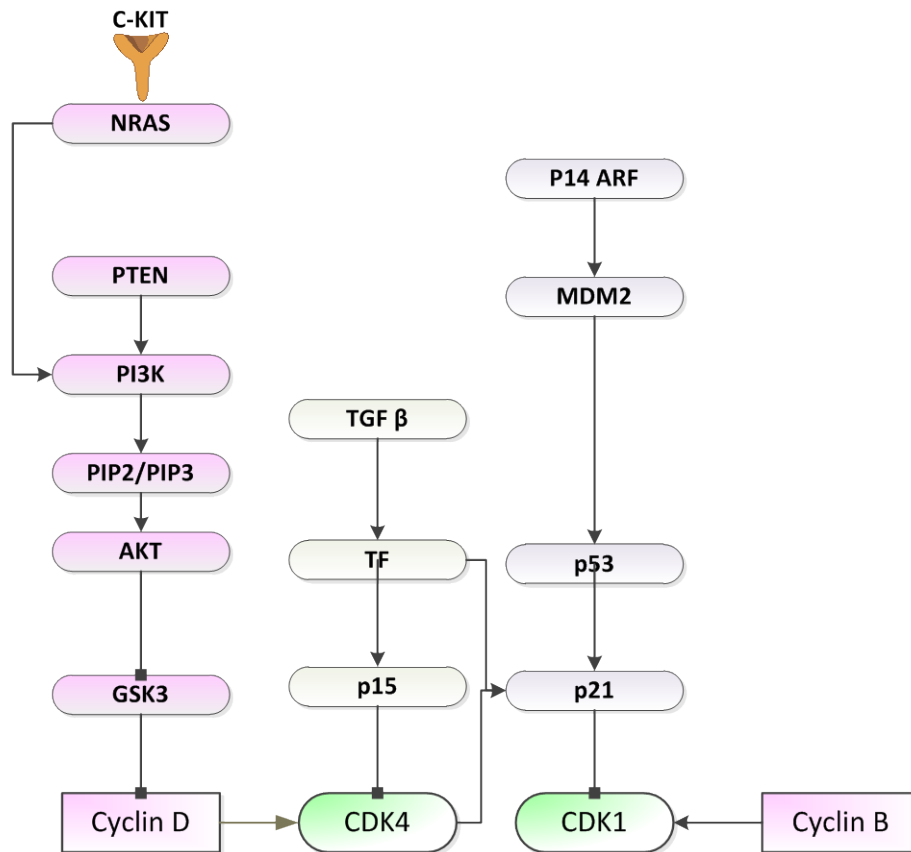
| <i>Gene</i> | <i>Location</i> | <i>Function</i> |
|---|-----------------|---|
| E2F1 ³⁹ (also RBP3; E2F-1; RBAP1; RBBP3) | 20q11.2 | The protein encoded by this gene is a member of the E2F family of transcription factors. The E2F family plays a crucial role in the control of cell cycle and action of 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 ⁴⁰ (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 ⁴¹ | 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. |

³⁹ <http://www.ncbi.nlm.nih.gov/gene/1869>

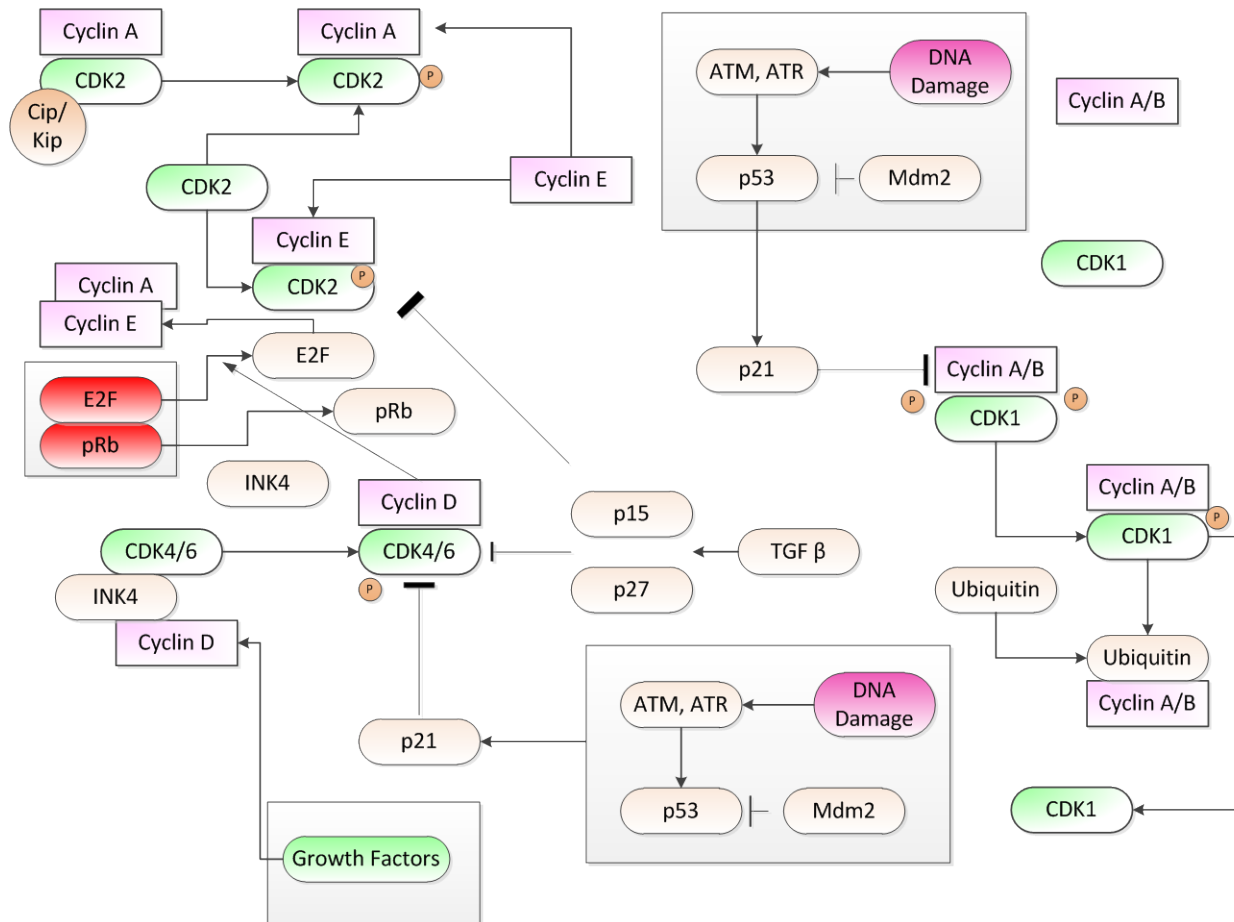
⁴⁰ <http://www.ncbi.nlm.nih.gov/gene/5925>

⁴¹ <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.

| <i>CKI Family</i> | <i>Member Name</i> | <i>Alternative Name</i> | <i>Gene</i> | <i>Function</i> |
|-------------------|---|-------------------------|-------------|--|
| INK4 Family | p15 ⁴² (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 ⁴³ (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. |

⁴² <http://www.ncbi.nlm.nih.gov/gene/1030>

⁴³ <http://www.ncbi.nlm.nih.gov/gene/1029>

| <i>CKI Family</i> | <i>Member Name</i> | <i>Alternative Name</i> | <i>Gene</i> | <i>Function</i> |
|-------------------|--------------------|-------------------------|-------------|--|
| | p18 ⁴⁴ | 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 ⁴⁵ | 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. |
| | | | | |

⁴⁴ <http://www.ncbi.nlm.nih.gov/gene/1031>

⁴⁵ <http://www.ncbi.nlm.nih.gov/gene/1032>

| <i>CKI Family</i> | <i>Member Name</i> | <i>Alternative Name</i> | <i>Gene</i> | <i>Function</i> |
|--------------------------|---|--------------------------------|--------------------|---|
| Cip-Kip Family | p21 ⁴⁶ 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 ⁴⁷ 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. |

⁴⁶ <http://www.ncbi.nlm.nih.gov/gene/1026>

⁴⁷ <http://www.ncbi.nlm.nih.gov/gene/5715>

| <i>CKI Family</i> | <i>Member Name</i> | <i>Alternative Name</i> | <i>Gene</i> | <i>Function</i> |
|-------------------|--|-------------------------|-------------|--|
| | p57 ⁴⁸ 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 syndrome, suggesting that this gene is a tumor suppressor candidate. |

The following genes are elements of cell cycle control.

| <i>Gene</i> | <i>Location</i> | <i>Function</i> |
|-------------------|-----------------|---|
| Jun ⁴⁹ | 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 ⁵⁰ | 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. |

⁴⁸ <http://www.ncbi.nlm.nih.gov/gene/1028>

⁴⁹ <http://www.ncbi.nlm.nih.gov/gene/3725>

⁵⁰ <http://www.ncbi.nlm.nih.gov/gene/2353>

| <i>Gene</i> | <i>Location</i> | <i>Function</i> |
|-------------------|-----------------|--|
| Myc ⁵¹ | 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 |

5.6 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 carcinogenesis⁵². 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.

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...

⁵¹ <http://www.ncbi.nlm.nih.gov/gene/4609>

⁵² Solimini, N., et al, Recurrent Hemizygous Deletions in Cancers May Optimize Proliferative Potential, Science, 6 JULY 2012 VOL 337, p 104.

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.*

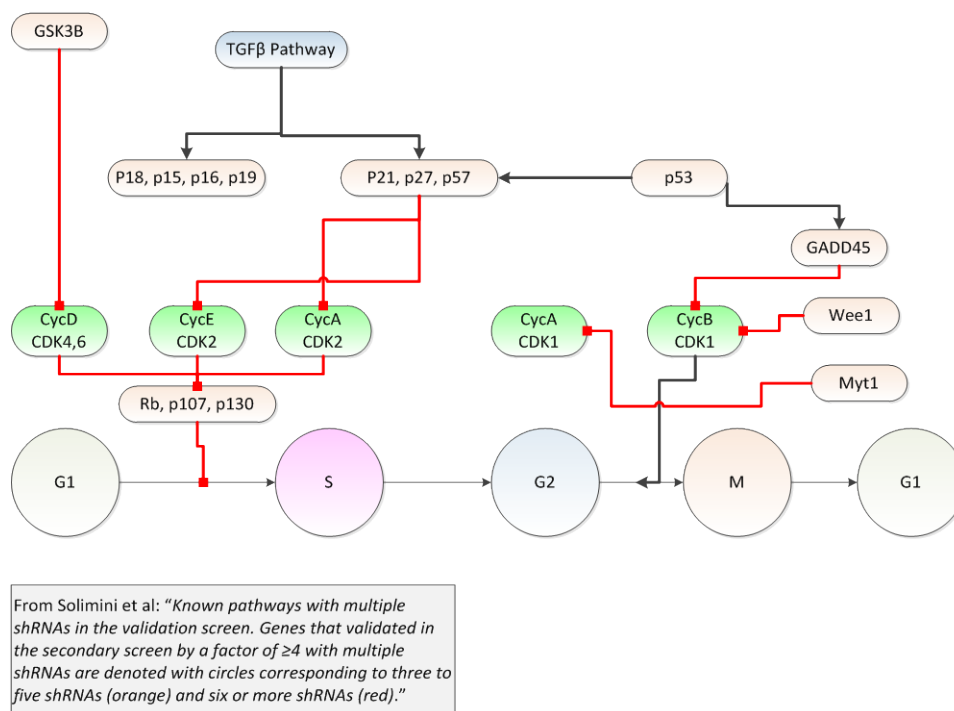
Because hundreds to thousands of genes are hemizygously deleted per tumor, this mechanism may help to drive tumorigenesis across many cancer types.

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 modified, and also not that they have short hairpin RNAs (shRNAs)).



They conclude as follows:

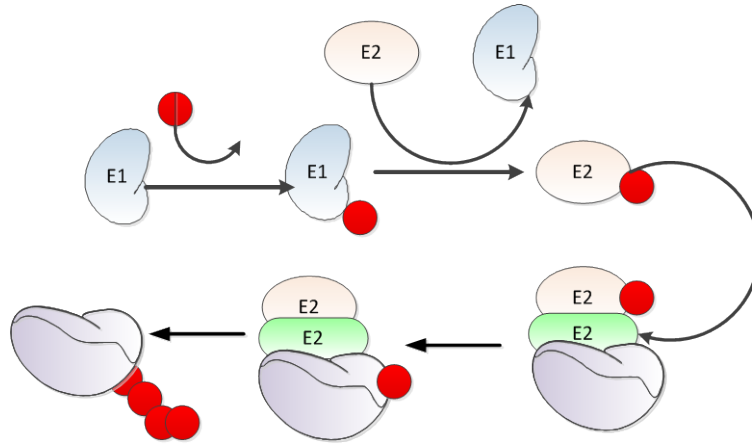
The enrichment for genes localized to deletions suggests that we have identified dozens of new TSGs in recurrent deletions. We have also likely identified more TSGs outside of these regions because the STOP gene set is (i) enriched for known TSGs, many of which are not found in recurrent deletions, and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

5.6.1 Ubiquitination

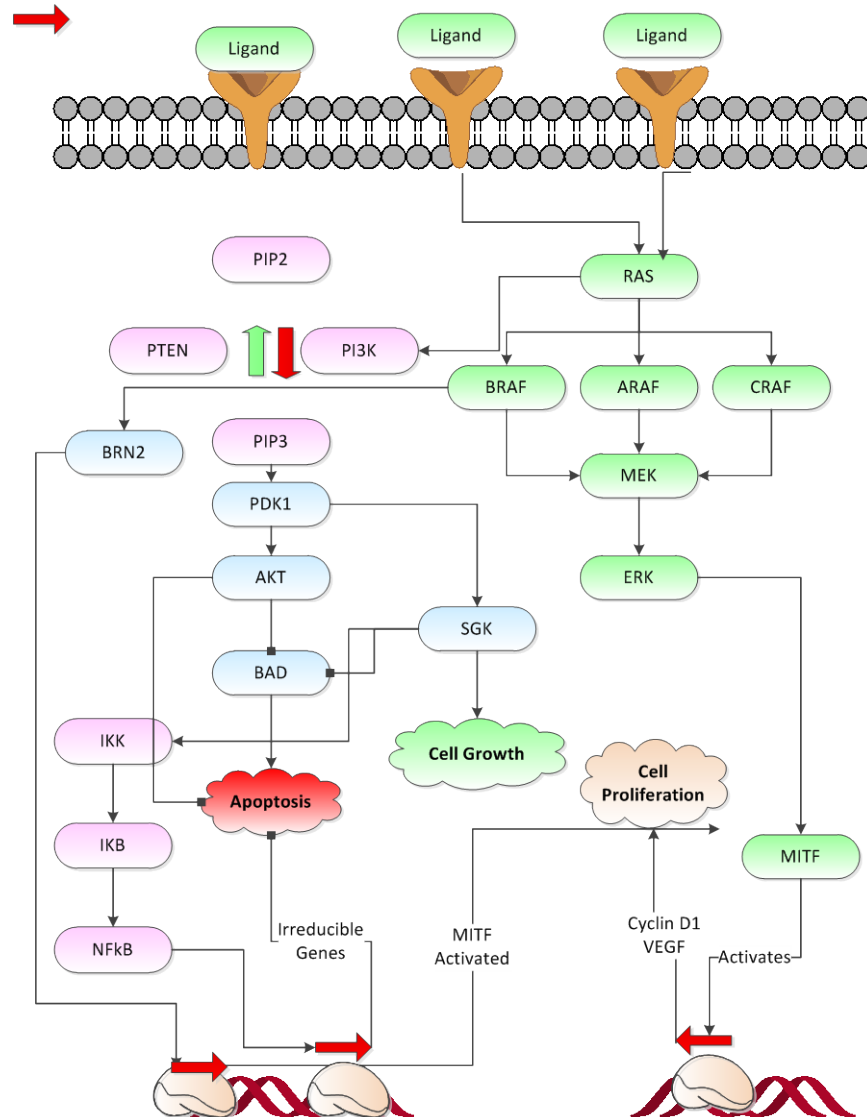
Ubiquitin is a small protein which acts with three related proteins; E1, E2, and E3. E1 is also called the ubiquitin activating enzyme, E2 the ubiquitin conjugating enzyme, and E3 ubiquitin ligase. Together they act to attach ubiquitin to a target protein and mark it for digestion and elimination. The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



Ubiquitination is an essential process within a cell to eliminate used or excess proteins. Although we will not discuss this in detail, it is an essential process and the reader should refer to standard texts⁵³.

The following Figure depicts some of the mechanics in terms of genetic flow and control as to how Ubiquitination occurs.

⁵³ See: Cassimeris et al p 688, Weinberg, p 242, Alberts et al, p 1065.



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? If we first examine skin cells, one of the many cells in the body which reproduce all the time, like blood cells, we can gain some insight.

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.

Now what of prostate cells, they do not reproduce as quickly. The glands are generally stable and often reproduce after some nominal lifetime of the basal or luminal cell. However a cell is stressed, for example by some external driver as inflammation, or other external attack, and then the cells may regenerate and thus reproduce. Perhaps that is one of the mechanism which underlies indolent PCa. Melanoma for example is highly aggressive in any form, most likely driven by the aggressive growth medium. However, as is known, melanocytes alone are indolent. This is one of those “on the one hand, on the other hand” arguments.

5.6.2 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. There have been a few studies regarding the kinetics, namely what facilitates and accelerates the cell cycle but there does not appear at this time to be a definitive conclusion.

5.7 SUMMARY

We have presented a high level summary of the DNA activity and the resulting cell cycle in mitotic activity. The cell cycle play an important role in cancer since inherent in any cancer is uncontrolled cell reproduction. The cyclins are at the heart of that process. It will be useful to go back to these basic ideas from time to time yet we do not consider the cell cycle as an integral part of our control model. Generally we try to take actions which prevent it from ever being entered. However it may become more critical to examine the cell cycle as a control point.

6 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.

This Chapter will be a key element in that we examine the intracellular pathways which control cell behavior.

6.1 PATHWAY OVERVIEW

We can now progress to aligning the gene products in an organized fashion, call pathways. The cell undergoes a continual process of generating various proteins and interacting with its environment through sending out proteins and by receiving and acting on them. The input-output process many in most cases be via ligands on the surface. During the normal homeostatic state of a cell, it is just acting like a small production factory reading its DNA and creating products. Changes may occur during this homeostatic state resulting from the attachment of miRNA, micro RNAs, or through the methylation of the cytosines on certain DNA strands. This then may result in a change during the static state. The cell also undergoes changes in the dynamic state of mitosis.

Our analysis will be general in nature but we will also provide some focus on specific PCa pathway concerns. Pathways as currently understood and presented represent the sets of gene expressions, namely the proteins produce by genes, and their gross effects on one another. For example in PCa we find that PTEN on Akt and then the effects on the gene, such as c-Myc on expression enhancements are often key to understanding the loss of normal homeostasis. In reality the gene is continually producing the proteins and their concentrations are often the drivers via the kinetics of reactions. Some proteins are catalysts to reactions to occur or to be blocked. Thus the true measures should be [PTEN], the concentration of PTEN, rather than just an on or off state. We shall discuss this later.

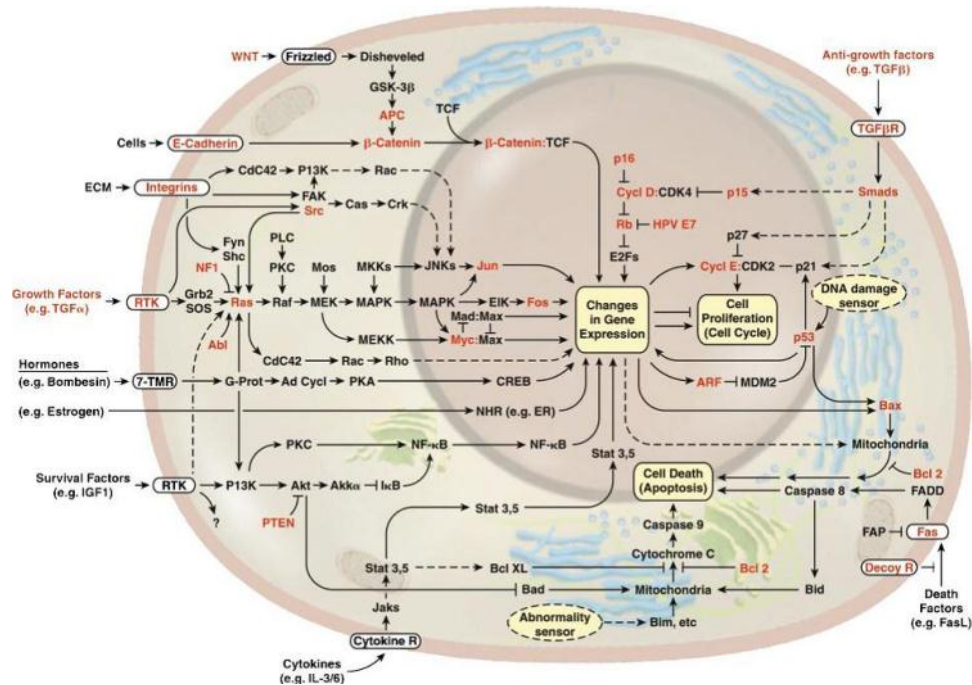
6.1.1 Global Cancer Pathway Models

Models for pathway elements and dependencies have been developed. The pathway models show:

(i) the cell wall and external signalling, including the possible ligands and receptors which can effect cell growth.

One of the standard set of pathways are presented below:

The end states of apoptosis versus proliferation are demonstrated. The issue is both gene activation, a binary issue of being present or absent, versus level of expression, how frequently is it being transcribed, are two separate but equally important considerations.



What is important to understand is that the above graphics are representative and fail to demonstrate the dynamics and the impact of protein concentrations and feedback on the evolution of a cell.

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.

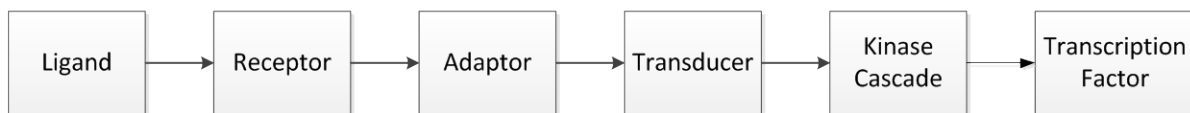
This is an input/output system. Namely, proteins in and proteins out, with proteins flowing internally in a complex control fabric. The goal of any cell is survival and proliferation. In cancer cells the cell manages to spread itself out with a total disregard for the rest of the cells in the body. We 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.

6.1.2 The Elements

We can envision the overall process by the model below where we categorize it by the element, and some examples:

| | | | | | |
|----------------------|--------------------------|------------------|----------------|--------------|-----------------|
| Ligand | PDGF | Insulin | Growth Hormone | IL-1 β | 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 |

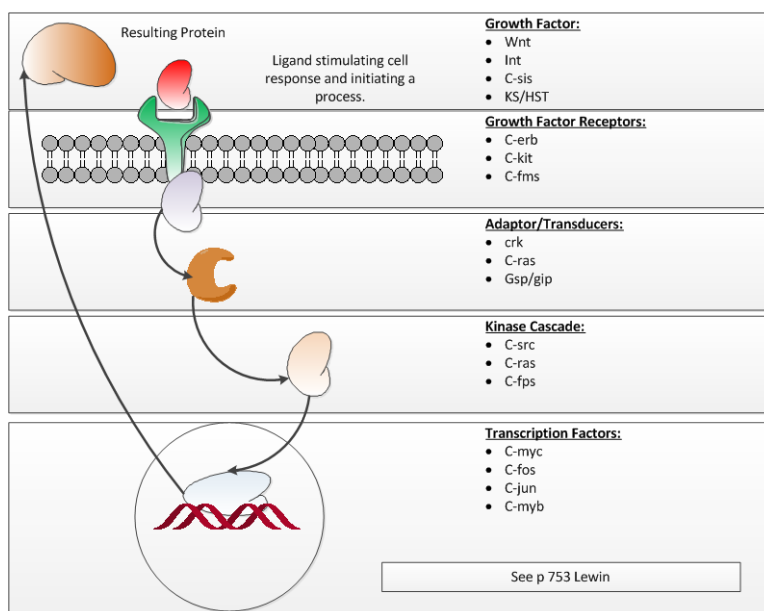
See p 818 Lewin



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

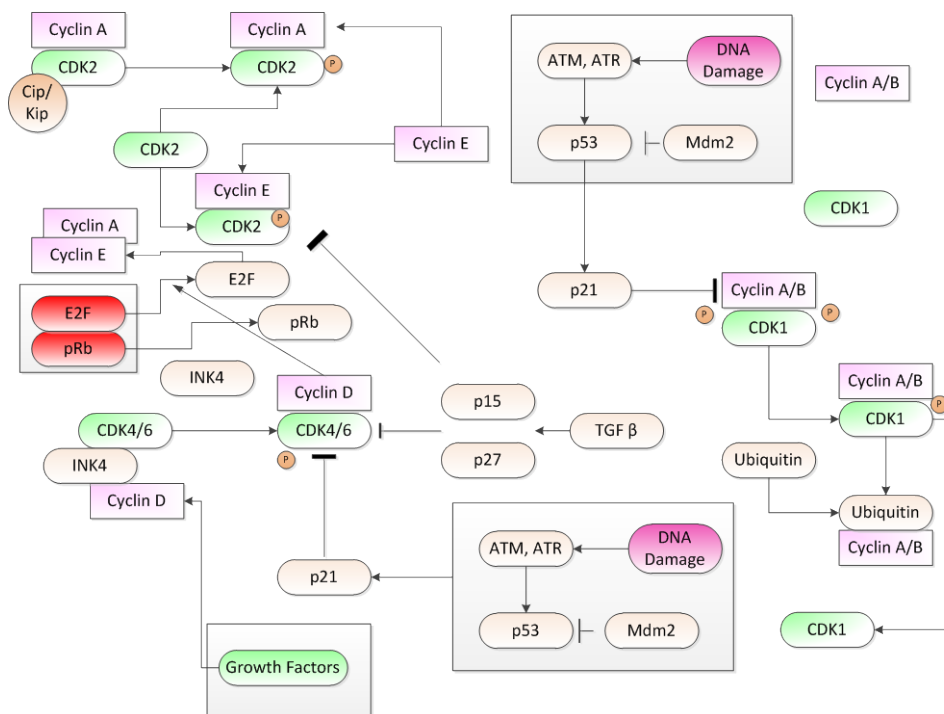
1. A ligand is generally a protein which is extra cellular. It finds its way to the cell outer membrane. We shall discuss this extracellular flow in some detail later. It may not be just some random diffusion but may have factors which direct its movement. This thus must become part of the overall modeling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



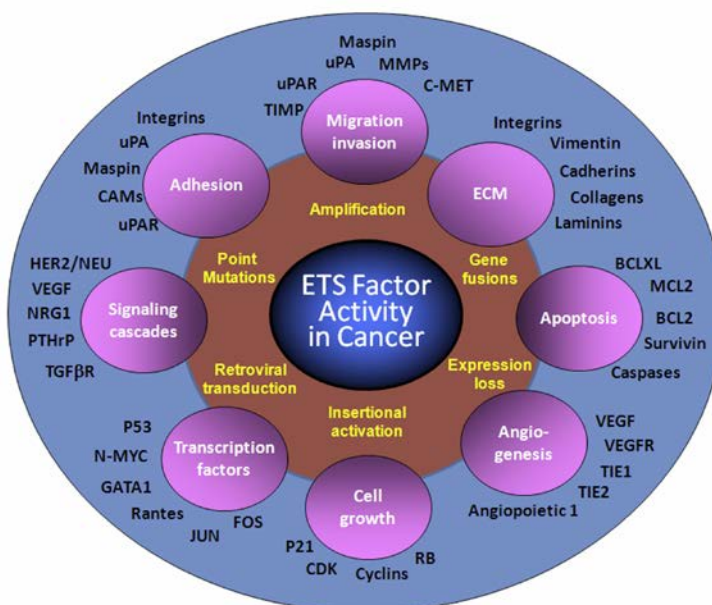
The question is; which genes do we focus on and why? In the chapter by Garraway and Chin in DeVita et al they present a Table of putative PCa genes (p 347). We repeat this table below:

| <i>Gene</i> | <i>Product Behavior</i> |
|-------------|-------------------------|
| 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 PCas. 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.

6.1.3 Specific Pathway Elements

A view of one set of factors, the ETS factors, by Watson et al depicts another view centered on a specific set of genes as seen below:



It should be noted that we did discuss the TMPRSS2 and ERG fusion, a fusion with an ETS gene, yet neither is depicted in the pathways shown above although they have a major role to

play in PCa. On the other hand we see from above that they affect many of the elements in that set of pathways.

One need just look at two factors below; first the TPMRSS2-ERG fusion and second loss of PTEN. The fusion is also noticed in PIN almost 20% of the time and in PCa almost 60% of the time. Is this causal or a result of the change? PTEN is not present in PIN but it is in PCa 60% of the time. The question that one must keep in the back of their minds is what is the dynamic model for this to occur.

The following Table is from Chen and Sawyers in DeVita and lists what they perceive as the key genes in the controlling pathways. We summarize them below and we have characterized them earlier.

| <i>Name</i> | <i>Type</i> | <i>Location</i> | <i>PIN Frequency</i> | <i>PCa Frequency</i> |
|--|---|-----------------|----------------------|----------------------|
| GST π Loss Glutathione S- transferases | Hypermethylated Gene | | 50% | 90% |
| TPMRSS2:ERG Translocation | Translocation | 21q22 | 0-20% | 50-60% |
| PTEN Loss | Loss | 10q23 | 0% | 60% |
| Akt (Akt1) | Pathway | 14q32.32 | | |
| NKX3.1 | Tumor Suppressor Homeobox TF | 8p21 | 60% | 85% |
| AR | Up-regulate | Xq12 | | |
| C-Myc | Transcription Factor | 8q24 | | ~90% |
| NF- κ B | Heterodimer of p65/p50 Transcription Factor | 10q24 | | |
| Wnt | Pathway | | | |
| Hedgehog | Pathway | | | |
| Notched | Pathway | | | |

6.1.4 Gene Specificity and Frequency

There has been a great deal of study of many genes which have been altered and thus reregulated in PCa. We rely upon the MSKCC portal which allows us to study their database of genes and PCa. We present a few cases here as examples and then we focus on a few which have received the greatest attention. The genes affected in the androgen pathway are shown below from the

MSKCC genomics data portal.⁵⁴ Specifically for the following cases we show the percent which have specific genes altered:

Case 1: Gene Set / Pathway is altered in 52.61% of all cases.

- Pathway: Prostate Cancer: AR Signaling (10 genes)
- Total number of input genes: 10
- Case Set: Prostate All: All prostate cancer samples (230 samples)
- Total number of cases selected: 230



Case 2: Gene Set / Pathway is altered in 80.87% of all cases.

- Pathway: Prostate Cancer: Sequenced (145 genes)
- Total number of input genes: 145
- Case Set: Prostate All: All prostate cancer samples (230 samples)
- Total number of cases selected: 230

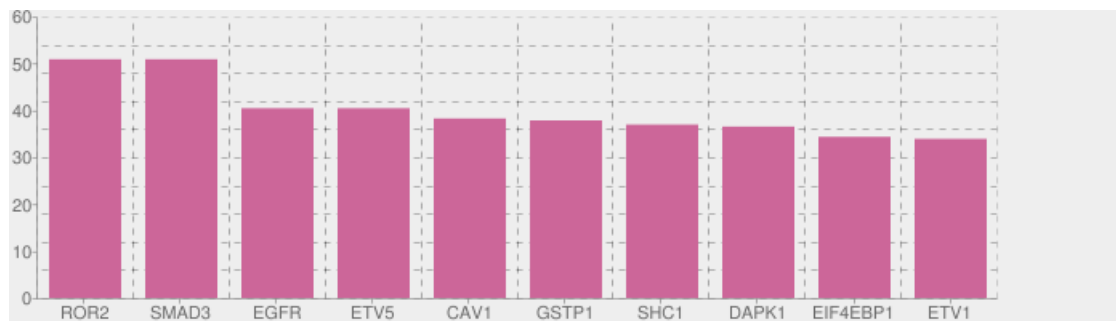


Case 3: Gene Set / Pathway is altered in 81.74% of all cases.

- Pathway: Prostate Cancer: Prostate cancer pathway (280 genes)

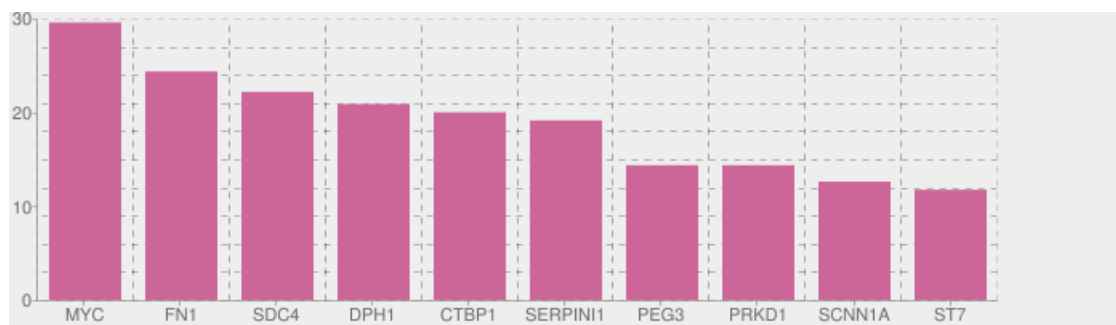
⁵⁴ http://cbio.mskcc.org/cancergenomics-dataportal/index.do#heat_map

- Total number of input genes: 280
- Case Set: Prostate All: All prostate cancer samples (230 samples)
- Total number of cases selected: 230



Case 4: Gene Set / Pathway is altered in 65.65% of all cases.

- Pathway: Prostate Cancer: Down-regulated by androgen (19 genes)
- Total number of input genes: 19
- Case Set: Prostate All: All prostate cancer samples (230 samples)
- Total number of cases selected: 230

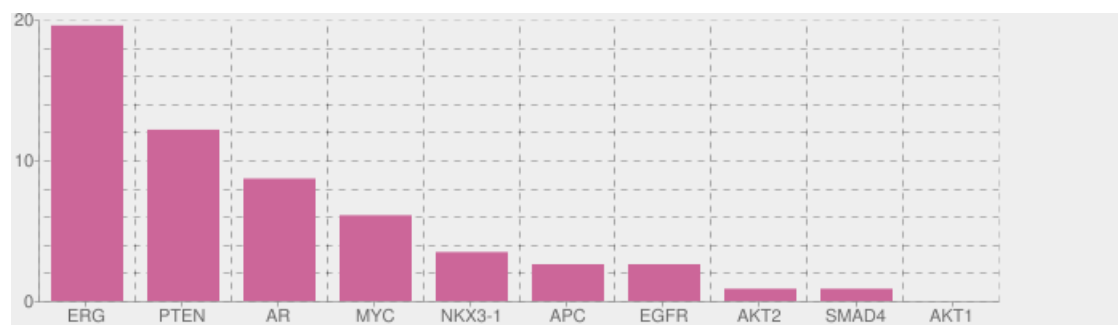


Case 5: Gene Set / Pathway is altered in 38.7% of all cases.

- Pathway: Custom
- Total number of input genes: 11
- Case Set: Prostate All: All prostate cancer samples (230 samples)
- Total number of cases selected: 230

| Case Id | ERG | PTEN | AR | MYC | NKX3-1 | APC | EGFR | AKT2 | SMAD4 | AKT1 | TMPRSS2 | Gene Set |
|-----------------------------------|---------|---------|-------|--------|--------|--------|--------|--------|--------|------|---------|----------|
| NUMBER OF CASES WITH ALTERED GENE | 45 | 28 | 20 | 14 | 8 | 6 | 6 | 2 | 2 | 0 | 0 | 89 |
| % OF CASES WITH ALTERED GENE | 19.57 % | 12.17 % | 8.7 % | 6.09 % | 3.48 % | 2.61 % | 2.61 % | 0.87 % | 0.87 % | 0 % | 0 % | 38.7 % |

We depict this data below:



We will focus on a few of the above specific genes; ERG, MYC, PTEN, AKT, AR, and TMPRSS2.

It is essential to remember the Dougherty discussion, namely one must not rely solely upon the gene and its presence, but one must understand how it functions, its relationship between itself and other genes, and the issues regarding over and under expression, and finally the issue of mutations and transpositioning. Also it is essential to keep the roles of miRNA in mind. Causality and coincidence must be determined in the development of models.

6.2 SOME SPECIFIC GENES

We will first consider several of the specific genes and their impact on the development of HGPIN and in turn PCa. In the next section we move on to the pathways and the interaction amongst and between the genes.

The genes and their resulting proteins fall into the following general categories⁵⁵:

Oncogenes: Oncogenes are mutated forms of a normal gene called a proto-oncogene. The proto oncogene is normally controlling or regulating the growth and the regulation of a cell. When changed it results in loss of control or excess growth and expansion. Some typical oncogenes are RAS, C-MYC, CDK4, ERBB2, MET, PIK3CA amongst many. For example C-MYC is a transcription gene, it facilitates DNA transcription. Normally its function is working in a controlled manner and transcription and the products are produced in a regulated concentration. However C-MYC can undergo changes which cause its amplification.

One manner of amplification is via amplification, a process where copies of the gene can be made and thus its product, c-myc protein, gets created at a greater concentration. Another change in C-MYC could be that of it being translocated and moved near another gene which amplifies the output as is seen in some leukemias.

Translocations: The movement of genes en masse from one chromosome to another is a translocation. The most prominent is the Philadelphia chromosomal translocation found in

⁵⁵ We will refer to Bunz for many of these descriptions.

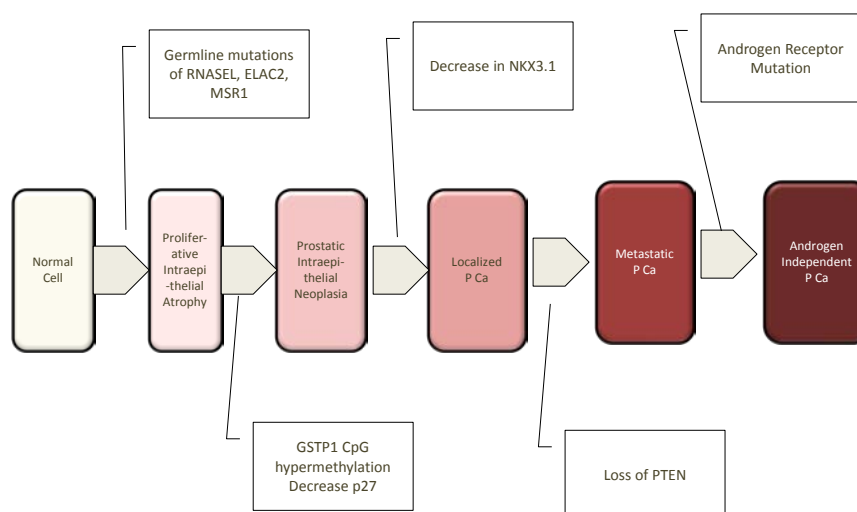
chronic myelogenous leukemia. In the case of PCa the translocation gene is the TMPRSS2:ERG fusion, which is in a sense a translocation since the intron between the two gene is removed and they are then fused.

Tumor Suppressor Genes: These genes are controllers of a set of processes key to the maintenance of the normal integrity of the cell. Tumor suppressor genes are frequently inactivated by mutations and it is their loss of function which results in cell instability and thus cancer. Typical Tumor Suppressor Genes, TSP, are TP53, or as it is known p53, which controls cell growth. PTEN is a second, and a key player in PCa. BRCA1 and BRCA2 are genes which are predominant in breast cancer. CDKN2A (also known as P16, INK4) is a key player in melanoma.

In this section we concentrate on those genes as characterized above which have the most significant role to play in HGPIN and PCa.

6.2.1 Genes and HGPIN

We now look at the putative genetic changes from benign prostate cells to HGPIN. The arguments in the literature for the most part state that the process is a result of genetic changes and that furthermore, implied but not expressly stated, these changes are immutable and they naturally progress from HGPIN to PCa. It should be noted that there does not appear to be any work indicating the regression of HGPIN as regards to specific genes or subsequently specific pathways. We start from the paper by Nelson et al (2003) where they argue that the genes we discuss below are the ones leading to full androgen independent PCa. We start with a modification of the process as described by Nelson et al. below:



From: Nelson W et al. N Engl J Med 2003;349:366-381

12/22/2010

17

They argue that to reach HGPIN it is necessary to go through a PIA step, proliferative intraepithelial atrophy, step and then full HGPIN. We now go through the first two steps and examine the genes related thereto. It should be recalled, however, that the Nelson model is but one of several. It is not as simple as the Vogelstein model for colon cancer. We shall return to a collection of these models later in this paper.

Thus we can reach the following conclusions from Nelson et al:

1. To reach PIA we need germline alterations of RNASEL, ELAC2, and MSR1.
2. To reach HGPIN we need hypermethylation of GSTP1 and a decrease in p27.

The steps towards PCa we will also discuss briefly. There are a few, clearly NKX3.1 is essential as is loss of PTEN and amplification of c-myc. The questions we ask are:

1. Can we get HGPIN without PIA as an initial step. Is hypermethylation alone needed. Also given the Goldstein model, where does Akt and PTEN suppression play in this process.
2. Is PIA regressible itself and if so is it due to apoptosis of the PIA cells and if so what causes the apoptosis, is it a normal cell death or exogenous to the cell say from the immune system.
3. If hypermethylation of GSTP1 is necessary for HGPIN, what causes its regression. What is one to make of the Goldstein model of Akt and the trans-located ERG TMPRSS change as well.

These are but a few of the issues which need be addressed.

6.2.2 PIA

The understanding of the development of PIA is still fragmentary. PIA is the purported first step. To Nelson, this requires first a germline mutation of RNASEL, ELAC2 and MSR1. Let us examine each of these in turn.

RNASEL: A gene which encodes a widely expressed latent endoribonuclease that participates in an interferon inducible RNA-decay pathway that is thought to degrade viral and cellular RNA.

What mutations, what causes the mutations, and what the cell response is to the mutations is still an open issue.

6.2.3 PIN

Now PIN is a more complex issue. The problem here is twofold. First, looking at human HGPIN and then performing microarray analysis one obtains many different profiles of genes expressed, under-expressed, or over-expressed. Second, there are murine models and other models in which specific genes are activated, changed or deactivated and where the relationship between murine models do not map one to one onto the human facts.

Morphologically and histologically we can identify HGPIN. It is simply the overgrowth within benign glands or acini of new cells whose characteristics appear to be neoplastic but whose growth is constrained. We see this in other areas such as melanoma in situ where the melanocyte may leave the basal layer and migrate to the upper epidermis or where the melanocyte may form clusters at the junction which are larger than say a junctional nevus, but true malignancy is not identifiable.

Let us briefly look at the genes involved in PCa. First there are the susceptibility genes which set the stage and although not drivers do facilitate the process. These are shown below, the Prostate-Cancer–Susceptibility Genes.

| Gene | Location | Alterations* | Phenotypic Consequences |
|---------------|----------|---|---|
| <i>RNASEL</i> | 1q24–25 | Base substitutions leading to Met1Ile, Glu265X, and Arg462Gln alleles Four-base deletion at codon 157 leading to premature protein truncation at codon 164 | Encodes endoribonuclease that participates in an interferon-inducible 2',5'-oligoadenylate– dependent RNA-decay pathway <i>RNaseL</i> ^{-/-} mice have diminished interferon- α antiviral activity |
| <i>ELAC2</i> | 17p11 | Base insertion leading to premature termination 67 amino acids after codon 157; base substitutions leading to Arg781His, Ser217Leu, and Ala541Thr alleles | Unknown |
| <i>MSR1</i> | 8p22 | Base substitutions leading to Arg293X, Pro36Ala, Ser41Tyr, Val113Ala, Asp174Tyr, Gly369Ser, and His441Arg alleles | Encodes subunits of class A macrophage- scavenger receptor <i>Msra</i> ^{-/-} mice have an increased sensitivity to serious infection with <i>Listeria monocytogenes</i> , <i>Staphylococcus aureus</i> , <i>Escherichia coli</i> , and herpes simplex virus type 1 |
| <i>AR</i> | Xq11–12 | Polymorphic polyglutamine (CAG) and polyglycine (GGC) repeats | Encodes androgen receptor, an androgen- dependent transcription factor Different polymorphic alleles may be associated with different transcriptional transactivation activities |
| <i>CYP17</i> | 10q24.3 | Base substitution in transcriptional promoter (T ⁺ C transition leading to new Sp1 recognition site) | Encodes cytochrome P-450c17a, an enzyme that catalyzes key reactions in sex-steroid biosynthesis |
| <i>SRD5A2</i> | 2p23 | Base substitutions leading to Val89Leu and Ala49Thr alleles | Encodes the predominant 5- α -reductase in the prostate, converts testosterone to dihydrotestosterone |

Now we can look at the genes modified that result in PCa. We list them below as the Somatic Gene Alterations in Prostate Cancer:

| Gene | Location | Alterations | Phenotypic Consequences | Initiates |
|---------------|----------|--|--|---|
| <i>GSTP1</i> | 11q13 | CpG island hypermethylation (decreased expression) | Encodes carcinogen-detoxification enzyme <i>Gstp1</i> ^{2/-} mice show increased skin tumorigenesis when exposed to topical carcinogen | PIN |
| <i>NKX3.1</i> | 8p21 | Allelic losses (decreased expression) | Encodes a prostate-specific homeobox gene essential for normal prostate development <i>Nkx3.1</i> ^{+/-} and <i>Nkx3.1</i> ^{-/-} mice manifest prostatic hyperplasia and dysplasia | Localized PCa |
| <i>PTEN</i> | 10q23.31 | Allelic losses, mutations, probable CpG island hyper-methylation (decreased expression, function, or both) | Encodes a phosphatase active against protein and lipid substrates <i>Pten</i> ^{+/-} mice have prostatic hyperplasia and dysplasia Prostatic intraepithelial neoplasia develops in <i>Pten</i> ^{+/-} <i>Nkx3.1</i> ^{+/-} and <i>Pten</i> ^{+/-} <i>Nkx3.1</i> ^{-/-} mice Prostate cancer with a poor prognosis develops in <i>Pten</i> ^{+/-} <i>TRAMP</i> mice | Initiates Metastatic PCa |
| <i>CDKN1B</i> | 12p12–13 | Allelic losses (decreased expression) | Encodes p27, a cyclin-dependent kinase inhibitor <i>Cdkn1b</i> ^{-/-} mice have prostatic hyperplasia Prostate cancer develops in <i>Pten</i> ^{+/-} <i>Cdkn1b</i> ^{-/-} mice | Reduced levels of p27, a cyclin-dependent kinase inhibitor encoded by the <i>CDKN1B</i> gene, also are common in prostate cancers, particularly in prostate cancers with a poor prognosis |
| <i>AR</i> | Xq11–12 | Amplification, mutations (increased expression, altered function) | Encodes androgen receptor <i>Pb-mAR</i> transgenic mice have prostatic hyperplasia, and prostatic intraepithelial neoplasia develops in them | Androgen Independent Metastatic PCa |

Yet to get to HGPIN Nelson et al argue we need hypermethylation of GSTP1 and a decrease in p27. Let us look at these two separately.

In the paper by Jeet et al (2010), the authors describe a set of genes which relate to HGPIN in detail based upon single trans-genic mice:

| Models | Genes regulated | Prostate phenotype |
|--|--|--|
| Hormone receptors | Androgen receptor | HGPIN |
| | Retinoic acid receptor α/γ | Squamous metaplasia and pre-neoplastic lesions |
| | Estrogen receptor α/β | No marked phenotype |
| Growth factors and receptors | FGF8b | HGPIN |
| | FGFreceptor1 | PIN with reversible hyperplasia |
| | FGF7 | Prostate epithelial dysplasia |
| | FGFR2iiib | Hyperplasia/dysplasia |
| | IGF-1 | PIN and spontaneous tumor growth |
| | TGFR- β | PIN and invasive adenocarcinoma |
| | HER-2/Neu | PIN and invasive carcinoma |
| | p53Rb | PIN with reduced apoptotic potential Focal hyperplasia |
| Tumor suppressors, cell cycle, and signaling pathways | Nkx3.1 | Hyperplasia followed by PIN |
| | H-Ras | LGPIN and intestinal metaplasia |
| | APC | PIN and invasive adenocarcinoma |
| | Pten | PIN and metastatic adenocarcinoma |
| | Bcl-2 | No overt phenotype |

| | | |
|----------------------------------|----------------------|---|
| | Akt-1 | Focal regions of PIN |
| | C-MYC | PIN and locally invasive adenocarcinoma |
| Genomic instability | Eco RI | HGPIN |
| | c-fos | No significant pathology |
| Composite transgenic mice | Ink4a/Arf+/-/Pten+/- | Rapid growth of PIN lesion |
| | Nkx3.1/Pten | PIN and metastatic spread of invasive tumors to lymph nodes |
| | Pten+/-/Akt1-/- | Akt1-/- repressed prostate tumor growth |
| | Pten+/-/p27kip1-/- | Rapid progression of invasive carcinoma |
| | Pten-/-/p53-/- | Early onset of invasive tumors |
| | PTEN+/-/TRAMP | Increased rate of tumor development |
| | P53-/-/Rb-/- | Highly metastatic adenocarcinoma |
| | Pten+/-/FGF8b | Metastatic adenocarcinoma |
| | Bcl-2/TRAMP | Multi step prostate carcinogenesis |

In the above studies there is a set of regressions noted. However, the study does make clear that there are multiple effects with limited consistency. They then continue to detail PCa genetic elements in the following Table.

| <i>Prostate phenotype</i> | <i>Models</i> | <i>Genetic disruptions</i> |
|---------------------------------|--|--|
| Hyperplasia | PB-FGF7(PKS) C3(1)-bcl-2PSA-CRExNkx3.1f/f PB-FGFRiiiib C3(1)-Polyomavirus Middle T MMTV-wap PB-Cre+/Rb loxp/loxP | FGF7 Bcl-2Nkx3.1 FGFRiiiib Polyomavirus middle T gene Whey acidic protein gene Retinoblastoma |
| PIN | ARR2PB-FGFR1 BK5-IGF1 ARR2PB-myc-PAI MPAKT model PSA-Cre+ <i>x</i> Nkx3.1+/flox PB-RAS PB-Cre4 <i>x</i> PtenloxP/loxP PB-Eco RI LPB-Tag/PB-Hepsin TRAMP LADY | FGFreceptor1 IGF-1 Myc Akt1 Nkx3.1 H-Ras PTEN ECO:RI Hepsin,p53,Rb p53,Rb p53,Rb |
| HGPIN | PB-m AR ARR2PB-FGF8b PB-Cre4 <i>x</i> PtenloxP/loxP MMTV-Crex PTEN loxp/loxP | Androgen receptor FGF8b Pten Pten |
| Locally invasive adenocarcinoma | C3(1)-SV40T/t PB-Cre+ <i>x</i> APCflox/flox PB/Neu | p53,Rb APC HER-2/Neu |
| Metastatic carcinoma | PSP-KIMAP Cryptidin-2/SV40T Fetal Gy-globin TRAMP PTEN+/-/TRAMP P53-/-/Rb-/- Nkx3.1-/-/Pten+/- Pten+/-/FGF8b | p53,Rb p53,Rb p53,Rb p53,Rb Pten,p53,RB p53,Rb Nkx3.1,Pten Pten,FGF8b |

Notwithstanding the complexity of the many elements above, the results do seem to bear some consistency on two issues; first, HGPIN has some regressible models, second, there are a common set of genes which do lead to PCa.

6.3 HGPIN GENES

For HGPIN there are two genes of focus, GSTP1 and p27, the product of the gene CDKN1B. We examine them as best understood today. The key question we keep in the back of our minds are what causes the change and then what causes a remission. Do all HGPIN have these genetic changes?

6.3.1 GSTP1

One of the first steps in the development of HGPIN it is alleged is the methylation of GSTP1 (GSTP1⁵⁶ (glutathione S-transferase pi 1, 11q13)). Glutathione S-transferases (GSTs) are a family of enzymes that play an important role in detoxification by catalyzing the conjugation of many hydrophobic and electrophilic compounds with reduced glutathione. Based on their biochemical, immunologic, and structural properties, the soluble GSTs are categorized into 4 main classes: alpha, mu, pi, and theta. This GST family member is a polymorphic gene encoding active, functionally different GSTP1 variant proteins that are thought to function in xenobiotic metabolism and play a role in susceptibility to cancer, and other diseases.

The methylation of sections of DNA has been seen as a major contributor to cancer formation. Regarding that of GSTP1, the work by Phe et al and that of Gonzalgo et al as well as Brooks et al demonstrate how the methylation is achieved and what the impact is. We will summarize their work herein.

As Deutsch et al state:

The GSTP1 enzyme acts as an reactive oxygen species scavenger. Inactivation of GSTP1 could lead to inefficiency During chronic inflammation processes, inflammatory cells generate oxidative reactive species that can produce genomic changes such as point mutations, deletions, and rearrangements.³⁵ Chronic inflammation involves repeated tissue damage and regeneration in the presence of highly reactive oxygen species that can interact with DNA. GSTP1 is both overexpressed and hypermethylated in about 10% of PIA.

The overexpression of GSTP1 and COX2 found in PIA suggest inflammatory and oxidative stresses in these cells.⁷³ A prostate-cancer progression model has been proposed, in which PIA is a precursor to prostate cancer via an intermediate stage of high-grade PIN. Like high-grade PIN and carcinoma, PIA occurs frequently in the peripheral zone of the prostate gland, and is often found in close proximity to carcinoma. Chromosome 8 changes have been found in microdissected samples of PIA at a frequency similar to that of high-grade PIN and prostate carcinoma.

⁵⁶ see http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene&cmd=retrieve&dopt=default&rn=1&list_uids=2950

Many PIA cells have decreased expression of P27, the cyclin-dependentkinase inhibitor, and highly express BCL2 as in high-grade PIN. PIA frequently shows morphological transition to PIN, and high-grade PIN occupies an intermediate position in the tumorigenic process with an intermediate phenotype harboring anomalies found in prostate cancer (table 3). However, these models are speculative without solid data confirming a defined role for either COX2 or GSTP1 as initiators of this process.

Deutsch et al (2004) also contains the following Table:

| | <i>Healthy Prostate</i> | <i>PIA</i> | <i>High-grade PIN</i> | <i>Prostate cancer</i> |
|------------------------------|-------------------------|------------|-----------------------|------------------------|
| COX2 | + | +++ | + | + |
| Morphological changes | 0 | -/+ | +++ | +++ |
| BCL2 | + | + | +++ | +++ |
| PCNA, KI67* | + | ++ | +++ | +++ |
| RER mutations | 4% | .. | 22% | 40% |
| Telomerase | 36% | 16% | 73% | .. |
| P27 | 86% | .. | 59% | 44% |

The contention is that there is a substantial change as one goes from benign to HGPIN. The reduction in p27 is significant and we discuss that later. The BCL2 and PCNA increased expression is interesting but it appears not to have been of continuing interest.

As DeMarzo et al states about prostate inflammation:

What is the source of prostatic inflammation? In most cases, the cause of prostatic inflammation is unclear. Various potential sources exist for the initial inciting event, including direct infection, urine reflux inducing chemical and physical trauma, dietary factors, estrogens, or a combination of two or more of these factors. Furthermore, any of these could lead to a break in immune tolerance and the development of an autoimmune reaction to the prostate.

However, there seems to be a consensus that stress, inflammation, oxidative stress, and the like has a precipitating set of factors for HGPIN. Donkena et al detail the oxidative stress arguments and Nelson et al detail those on inflammation. Deutsch et al provides an excellent summary of all the environmental and genetic factors. Palapattu et al provide an alternative view on the inflammation drivers. Ramoutar and Brumaghim details the use of antioxidants as means to counter these effects.

Specifically Brooks et al (1998) state:

In all prostate tissue specimens examined by immunohistochemistry, normal prostatic epithelia, particularly the basal cell layer, demonstrated the expression of GSTP1 immunoreactive polypeptide. All cancerous epithelia were devoid of GSTP1 expression...

Indeed, basal cells in each of the 60 high-grade PIN lesions could be identified by their abundant staining for GSTP1. Dysplastic epithelia (characterized by prominent nucleoli,

increased nuclear size, and multilayering of cells) demonstrated a complete absence of GSTP1 expression. Lack of expression occurred whether the PIN focus was adjacent to (30 cases) or far removed from (30 cases) prostate adenocarcinoma...

High-grade PIN lesions were evaluated for GSTP1 expression and GSTP1 promoter methylation because of their purported role as a prostate cancer precursor lesion.

Low-grade PIN (grade 1) has a more uncertain relationship to prostate cancer. Low-grade PIN is not associated with increased risk of prostate cancer on subsequent biopsy. Histological diagnosis of low-grade PIN is poorly reproducible even by experienced pathologists (10, 11). Many question whether low-grade PIN is related to high-grade PIN or prostate adenocarcinoma, or whether it is merely a normal epithelial variant.

High-grade PIN, on the other hand, is characterized by cytological and nuclear morphometric changes characteristic of carcinoma. Nearly 80% of prostates removed for adenocarcinoma harbor histological PIN lesions accompanying the primary tumor. Detection of high-grade PIN on prostate needle biopsy is associated with a 50% chance of detection of cancer on subsequent biopsy...

It is fair to state that the numbers in the 1998 Brooks et al article as stated above for HGPIN and PCa have dropped dramatically over the past decade or more.

6.3.2 CDKNA1B:p27

This gene protein, p27, encoded by gene CDKNA1B, encodes a cyclin-dependent kinase inhibitor, which shares a limited similarity with CDK inhibitor CDKN1A/p21. It is considered a tumor suppressor gene along with p53, PTEN and NKX3.1 (see Chen and Sawyers in DeVita, Cancer).

p27 is a cyclin-dependent kinase inhibitor and Cdkn1b^{-/-} mice, deficient of p27, are shown to have prostatic hyperplasia. Prostate cancer is shown to develop in Pten^{+/-} and Cdkn1b^{-/-} mice. The encoded protein binds to and prevents the activation of cyclin E-CDK2 or cyclin D-CDK4 complexes, and thus controls the cell cycle progression at G1. p27 is considered a tumor suppressor gene which can lose its effect early and be one of the drivers for HGPIN.

The degradation of this protein, which is triggered by its CDK dependent phosphorylation and subsequent ubiquitination by SCF complexes, is required for the cellular transition from quiescence to the proliferative state. p27 is a gene (located at 12p13.1-p12) involved in the control of kinase chains in the cell and controls cyclin for cell reproduction⁵⁷. In a recent paper by Majumder et al (2008) they state:

Transgenic expression of activated AKT1 in the murine prostate induces prostatic intraepithelial neoplasia (PIN) that does not progress to invasive prostate cancer (CaP). In luminal epithelial

⁵⁷ Also known as CDKN1B cyclin-dependent kinase inhibitor 1B (p27, Kip1) see http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene&cmd=retrieve&dopt=default&rn=1&list_uids=1027

cells of Akt-driven PIN, we show the concomitant induction of p27(Kip1) and senescence. Genetic ablation of p27(Kip1) led to down-regulation of senescence markers and progression to cancer. In humans, p27(Kip1) and senescence markers were elevated in PIN not associated with CaP but were decreased or absent, respectively, in cancer-associated PIN and in CaP. Importantly, p27(Kip1) up-regulation in mouse and human in situ lesions did not depend upon mTOR or Akt activation but was instead specifically associated with alterations in cell polarity, architecture, and adhesion molecules. These data suggest that a p27(Kip1)-driven checkpoint limits progression of PIN to CaP.

Weinberg describes the function of p27 in the cyclin-CDK complexes that regulate cell reproduction⁵⁸. Also the work of Yang et al describes the reduced expression of p27 as a major factor in the development towards PCa.

As DeMarzo et al state:

In terms of molecular modes of action, p27 functions as an inhibitor of cell-cycle progression by inhibiting the activity of cyclin–cyclin dependent kinase complexes in the nucleus. Interestingly, p27 levels are generally reduced but not absent in human proliferative inflammatory atrophy (PIA), prostatic intraepithelial neoplasia (PIN) and prostate cancer. The fact that p27 levels are not lost entirely (or biallelically inactivated by mutations) in cancer might be explained by recent findings that indicate that cytoplasmic p27 levels, which are increased by signalling through the MET receptor tyrosine kinase, are required for cell migration in response to hepatocyte growth factor signalling through MET and in response to increased cyclin D1 levels. Therefore, although high levels of nuclear p27 can prevent cell-cycle progression, cytoplasmic p27 might be required for optimal tumor cell motility, which is a key feature of malignant transformation and tissue repair.

There is the issue therefore as to what function p27 plays across many cancers. As Singerland and Pagano state:

CDKN1B cyclin-dependent kinase inhibitor 1B (p27, Kip1)

p27 is a cell cycle inhibitor whose cellular abundance increases in response to many antimitogenic stimuli. In this review, we summarize the current knowledge on p27 function and its regulation by synthesis and by ubiquitin-mediated degradation. Importantly, p27 degradation is enhanced in many aggressive human tumors. The frequency with which this is observed suggests that loss of p27 may confer a growth advantage to these cancers.

Thus across many types of cancer p27 is down regulated and allows uncontrolled growth since its control on the cyclins is removed.

In a recent paper by Taylor et al they conclude regarding murine models treated with 9-cis retinoic acid (9cRA), which had been shown to reduce the incidence of HGPIN in the murine models, that :

⁵⁸ Weinberg pp 268-279.

1. p27Kip1 deficiency increases prostate epithelial cell proliferation
2. p27Kip1 deficiency promotes prostate carcinogenesis
3. 9cRA suppresses prostate carcinogenesis irrespective of p27Kip1 expression
4. 9cRA was unable to suppress hormone-induced prostate carcinogenesis
5. 9cRA inhibits prostate cell proliferation and induces cellular senescence

They then conclude:

Our data indicate that p27Kip1 deficiency in PEC promotes cell proliferation in an age-dependent manner and increases cellular response to hormone stimulation. p27Kip1 haplo-insufficiency and deficiency stimulate MNU-induced prostate carcinogenesis, suggesting that PIN in human prostate lacking partial or total p27Kip1 expression may have a higher potential to progress and develop malignant phenotype than lesions with wild type p27. The efficacy of 9cRA in suppressing PIN is apparently not p27-dependent, indicating that in potential clinical trials, 9cRA may affect pre-malignant and tumor cells that differentially expressed p27Kip1. The induction of CS by 9cRA in PIN suggests that this biomarker could be used as a potential biomarker of response in clinical trials for the prevention and treatment of prostate cancer.

Thus a reduction in p27 expression is clearly related to HGPIN. The issue is what causes this underexpression. We will see later the prominent role p27 plays in the control but the details of its underexpression seem yet to be clarified.

As Deutsch et al state:

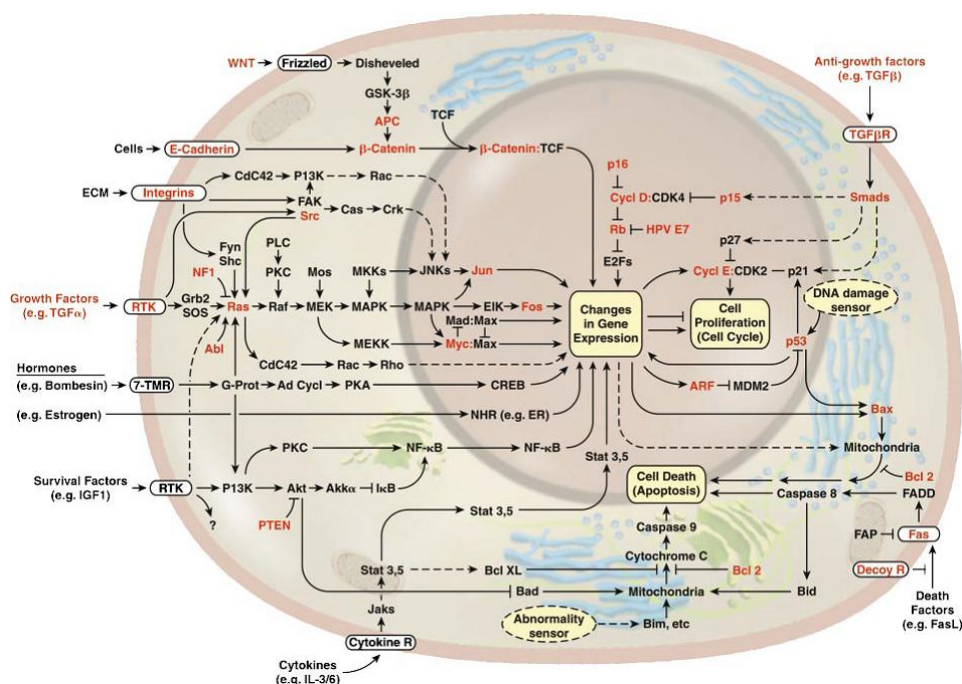
P27 is an inhibitor of the CDK2-cyclin E complex, thus regulating cell-cycle progression through the G1 cell cycle phase. A low concentration of P27 has been associated with the Gleason score and aneuploidy in studies based on primary prostate-cancer samples. Low expression of P27 is associated with higher recurrence and poor disease-free survival rates. One study found down-regulation of P27 in high-grade PIN samples, supporting the fact that P27 down-regulation could occur at early stages of carcinogenesis.

The important role of P27 in the development of the healthy prostate gland has been shown in p27 knockout mice, which have an enlarged prostate gland. The inactivation of one Pten allele and one or both alleles encoding p27 in mice was shown to accelerate spontaneous neoplastic changes showing that Pten and p27 have a pivotal and cooperative role in prostate cancer cell suppression. Moreover, this study strongly supports the theory of multistep carcinogenesis in prostate cancer, with the crucial relevance of a two-hit process (P27 and PTEN) occurring along the transformation process.

6.4 GENERIC OVERVIEW

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:

| | | | | | |
|----------------------|--------------------------|------------------|----------------|--------------|-----------------|
| Ligand | PDGF | Insulin | Growth Hormone | IL-1 β | 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. $\kappa\iota\upsilon\upsilon\omicron$ 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:

| <i>Gene</i> | <i>Product Behavior</i> |
|-------------|-------------------------|
| 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.

6.5 THE SINGLE CELL

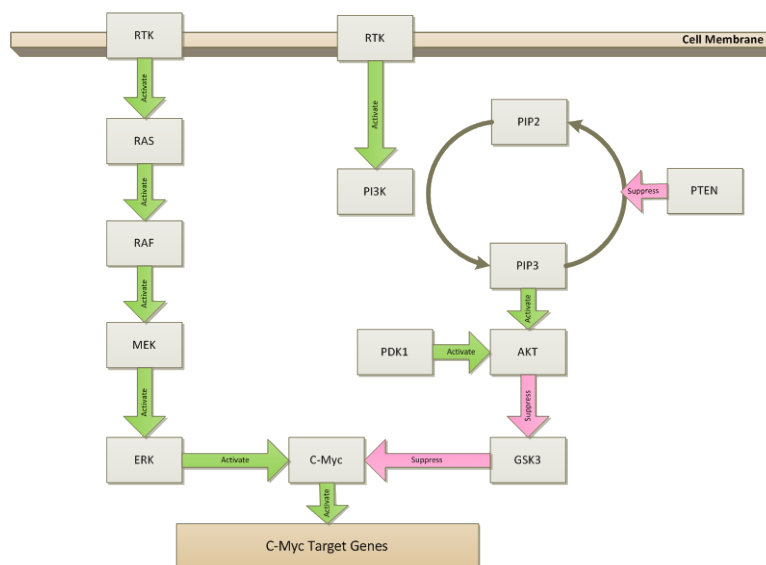
Let us begin with a single cell. We show this below in several simple pathway elements. Here we have three of the possible elements:

Receptors: This 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 are 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.

6.6 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 found in melanomas. These may be causative or they may be a result of the process. As Ugurel et 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

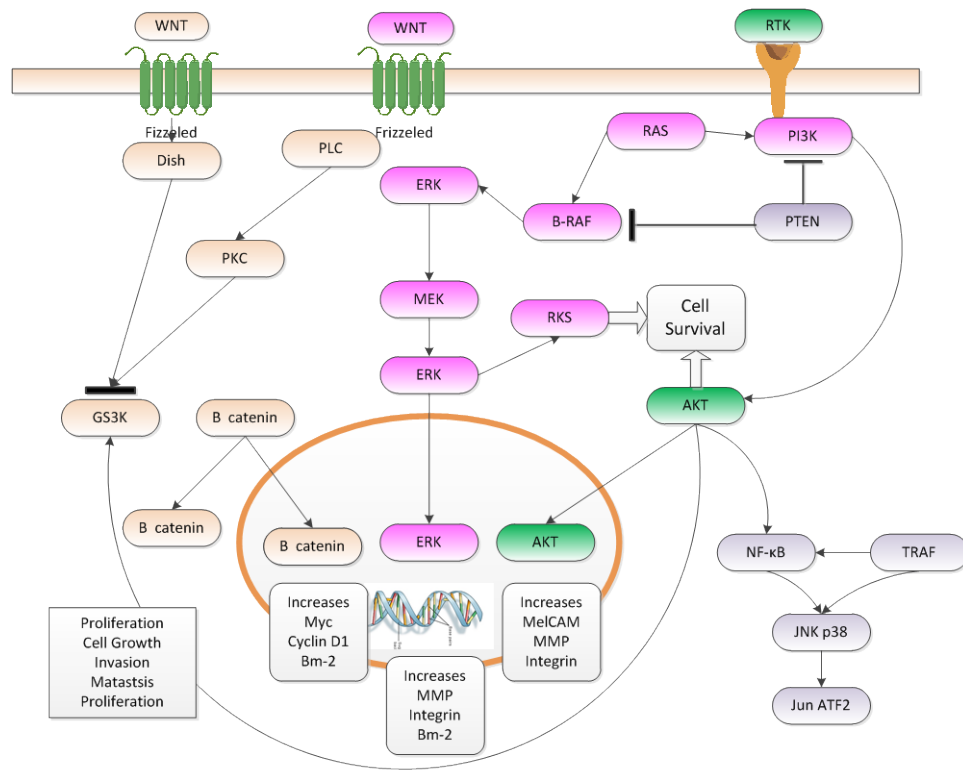
genes, 141 were underexpressed and 33 overexpressed in tumors whose host remained free of metastasis for 4 years. Of these 174 genes, 30 had been already studied in melanoma; these genes are involved in cell cycle regulation (CKS2, CDC2, CCNB1, CENPF, and DHFR), mitosis (HCAP-G and STK6), mitotic spindle 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 | | | | | | | | | |
| Dishevelled 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-3 β | 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 |
| MAPK | Ligand | | | | | | | | | |
| MEK | Pathway | | | | | | | X | | |
| MITF | Transcription | X | | | | | X | | | |
| mTOR | Pathway | | | | | | | | | |
| MYC | Transcription | | | | | | | | | |
| NEDD9 | Pathway | X | | | | | | | | |

| <i>Element</i> | <i>Type</i> | <i>DeVita et al</i> | <i>Hearing and Leong</i> | <i>Murphy</i> | <i>Bunz</i> | <i>Schulz</i> | <i>Vidwans et al</i> | <i>Fecher et al</i> | <i>Ugurel et al</i> | <i>Recent</i> |
|----------------|---------------|---------------------|--------------------------|---------------|-------------|---------------|----------------------|---------------------|---------------------|---------------|
| 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 | Pathway | | | | | | | | | |
| 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.

6.7 CELL SURFACE LIGANDS AND RECEPTORS

We begin with a discussion of several of the key receptors and ligands.

6.7.1 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

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 of receptors to the degree where we can include them in dynamic models.

6.7.2 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.

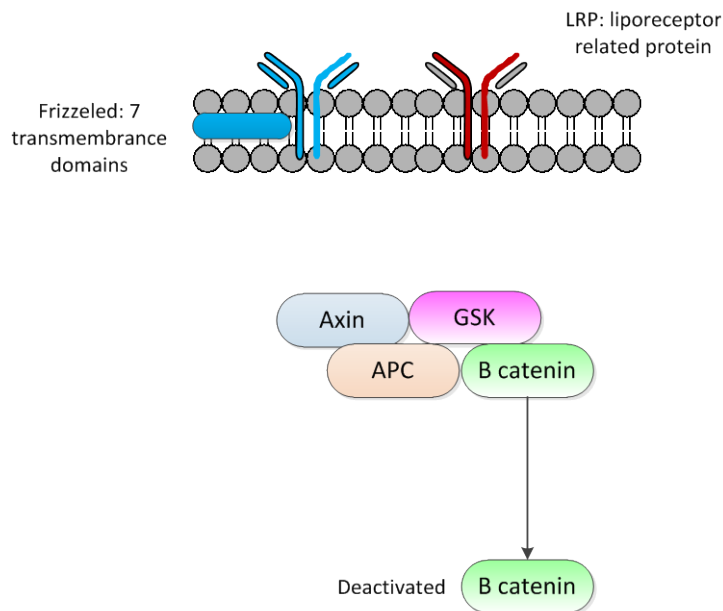
There are several other pathway elements that are worth commenting on. We do three here; WNT, Hedgehog, and Notched.

6.1.4.1 WNT

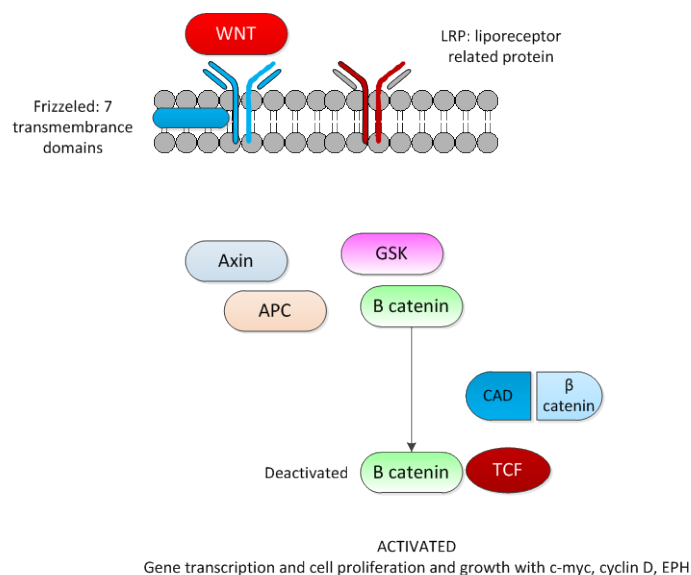
Wnt is characterized as follows:

- The name comes from the “wingless” gene and thus the Wn prefix. 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

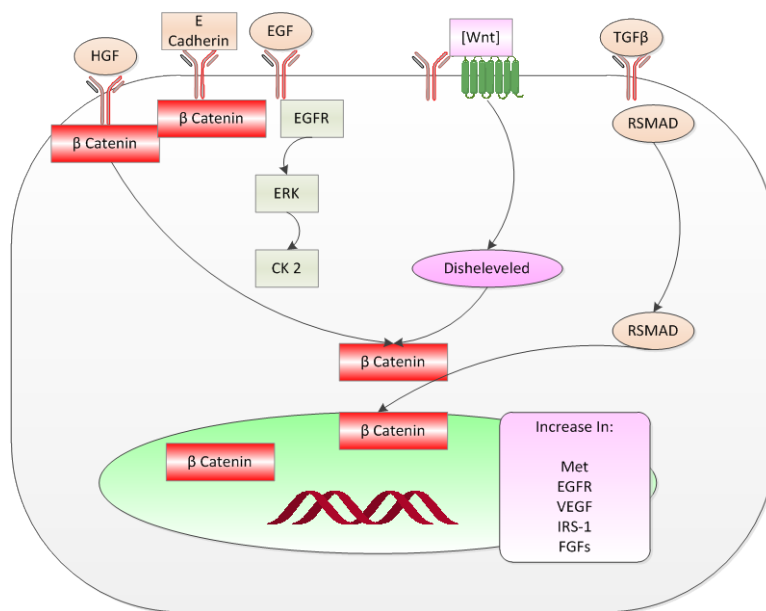
We depict this below, first the inactive state:



Then we depict the activated state with Wnt attached to the receptor:



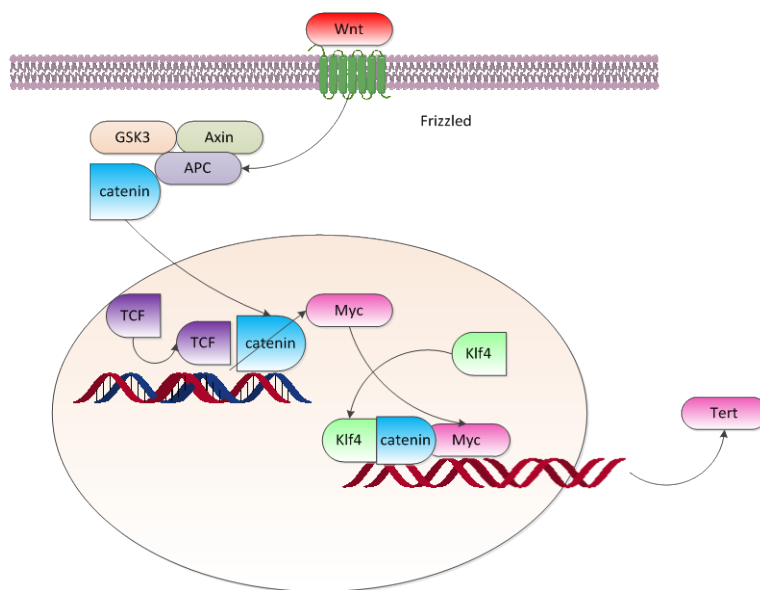
The figure below details many of the effects of Wnt binding:



6.1.4.2 *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 TERT⁵⁹.

⁵⁹ 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](https://www.ncbi.nlm.nih.gov/gene/7015)⁶⁰:

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:

⁶⁰ <http://www.ncbi.nlm.nih.gov/gene/7015>

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 κ B (NF- κ B), 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.

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.

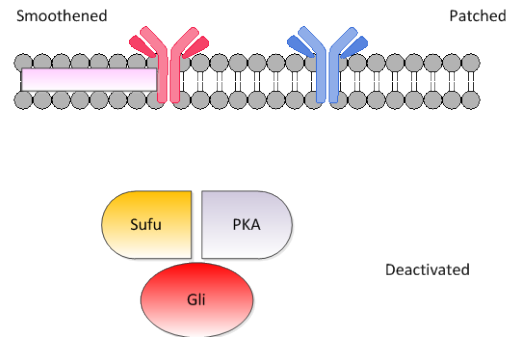
6.1.4.3 Hedgehog

Hedgehog is a ligand which activates receptors and then pathways. The Hedgehog pathway is also a key element characterized as follows:

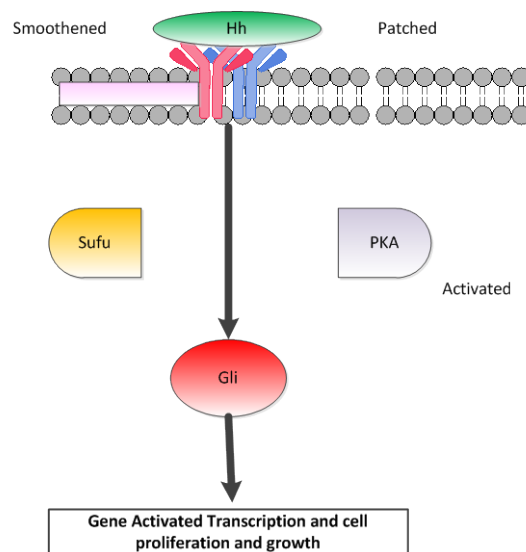
- In the absence of Hh a cell-surface transmembrane protein called Patched (PTCH) acts to prevent high expression and activity of a 7 membrane spanning receptor called Smoothened (SMO).
- Patched has sequence similarity to known membrane transport proteins. When extracellular Hh is present, it binds 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

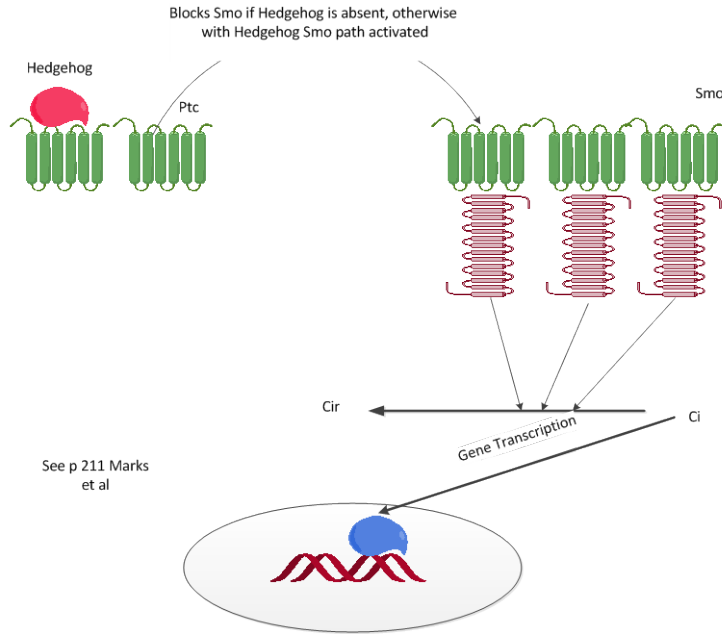
of this protein is negatively regulated by p53 in an inhibitory loop. Thus by activating Hh and combining the two receptors, Smo (Smoothened) and Patched (Ptch) we then activate Gli by unbinding it from Sufu and PKA. This is an example where we have three type at once; Wht the ligand, Smo and Ptch as receptors and Gli as a transcription factor.



Then the activated pathway as follows:

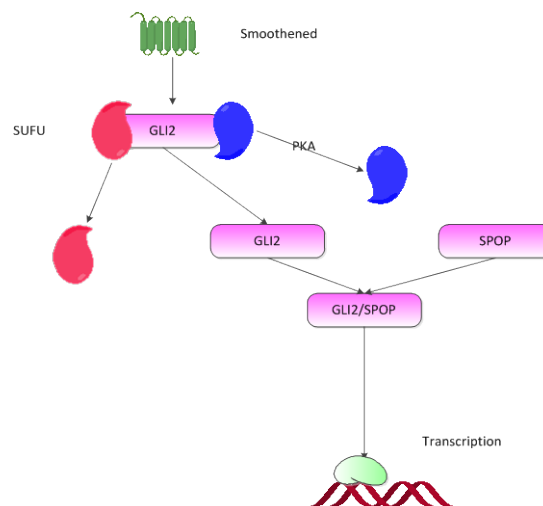


We demonstrate in more detail below the Hh binding. This graphically demonstrates the activation of the transcription factor and its movement into the nucleus and transcribing.



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, 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.

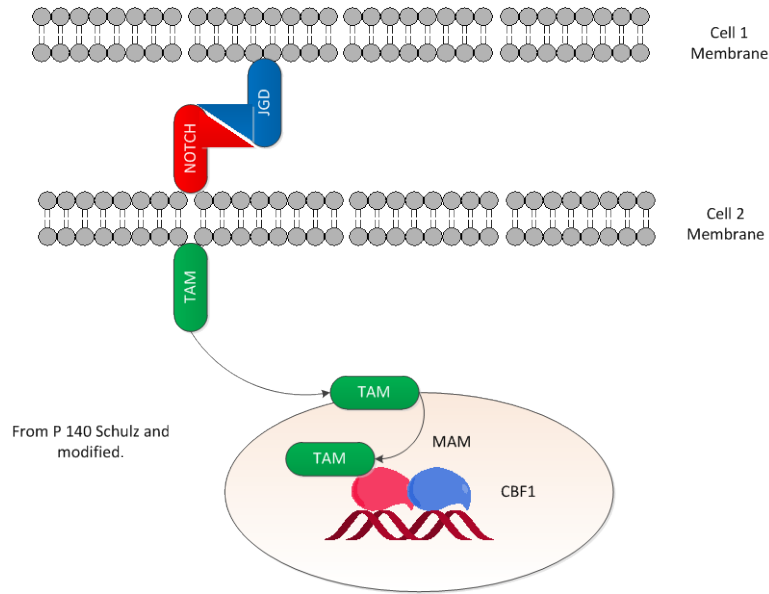
6.1.4.4 *Notched*

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

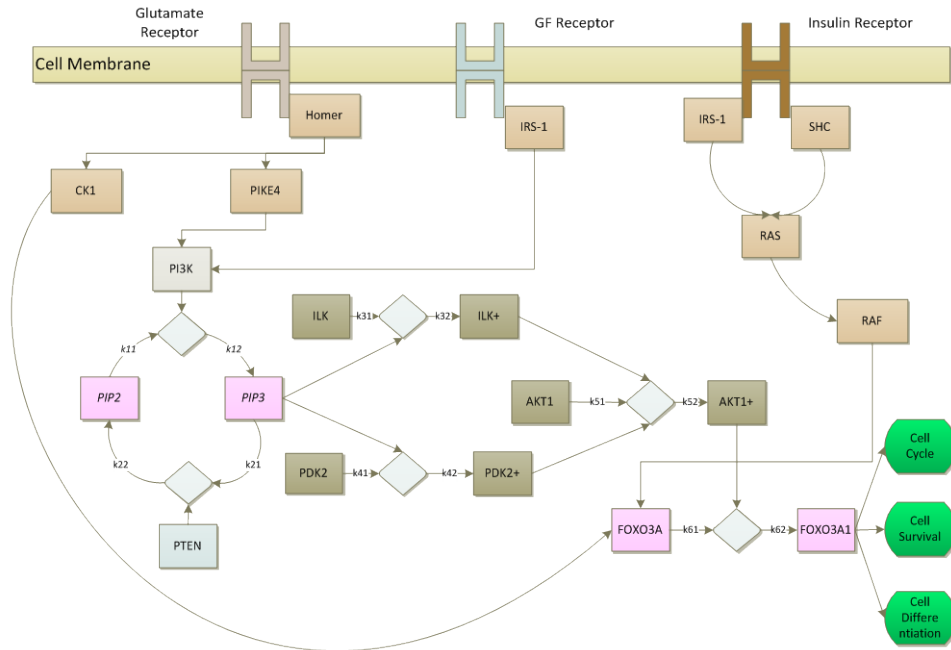
We demonstrate Notched and its counterpart Jagged in the following Figure. On the cell surface we have Notched and on the other cell surface we have Jagged. When they bond, in a sense as surface proteins but with a communicating capability, Notched releases or activates Tam which is a transcription factor facilitator.



Notch signaling is dysregulated in many cancers.

6.7.3 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.



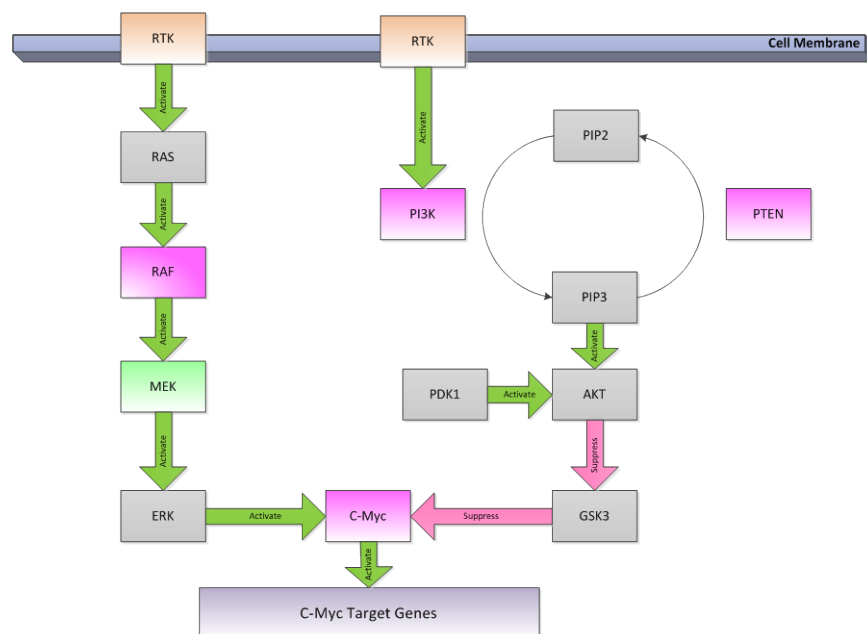
Note in the above we have demonstrated the move towards FOXO, a pathway element which activates cell growth, reproduction and transcription.

6.8 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.

6.8.1 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)P₃) and phosphatidylinositol-3,4-bisphosphate (PI(3,4)P₂).

Upon generation, PI(3,4,5)P₃ 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(3,4,5)P₃ and (PI(3,4)P₂), 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 of 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.

6.8.2 PI3K

As Davies states:

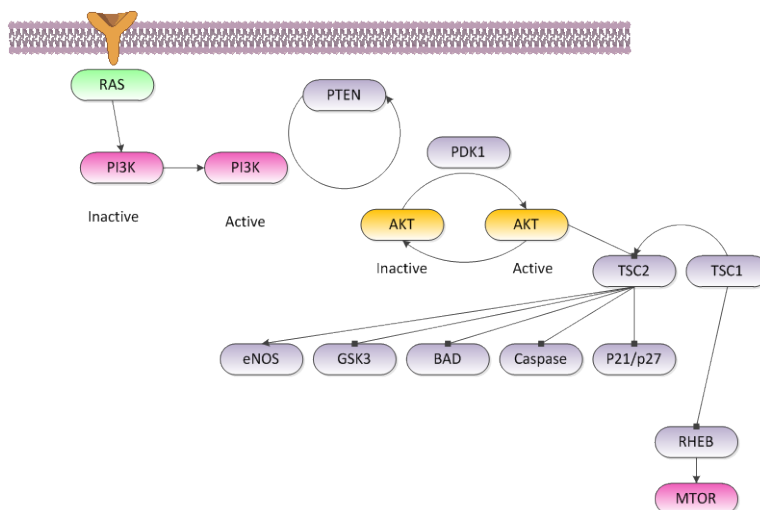
The PI3K (phosphatidylinositol 3-kinase)/AKT pathway is one of the most important signaling networks in cancer. There is growing evidence that activation of this pathway plays a significant role in melanoma, frequently in the setting of concurrent activation of RAS-RAF/MEK- ERK signaling. This evidence 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)/AKT 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. 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 there are several studies supporting a high concordance of activating BRAF and NRAS mutations in melanoma patients, data in other tumors types suggest that the PI3K-AKT 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:

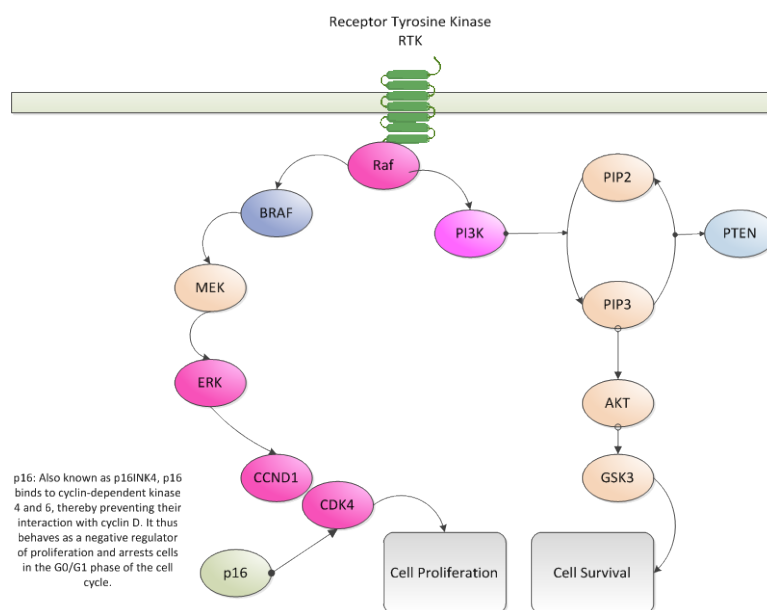
The high prevalence of activating BRAF and NRAS mutations in melanoma provided a strong rationale to test the effects of inhibitors against the RAS-RAF-MEK-ERK pathway in this disease. Multiple studies demonstrated that inhibition of the BRAF protein in human melanoma cell lines with small inhibitory 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 animal models.

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.

6.8.3 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 Kinase can get activated and Raf activates B-RAF which in turn activates MEK and then ERK then CCND1 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.



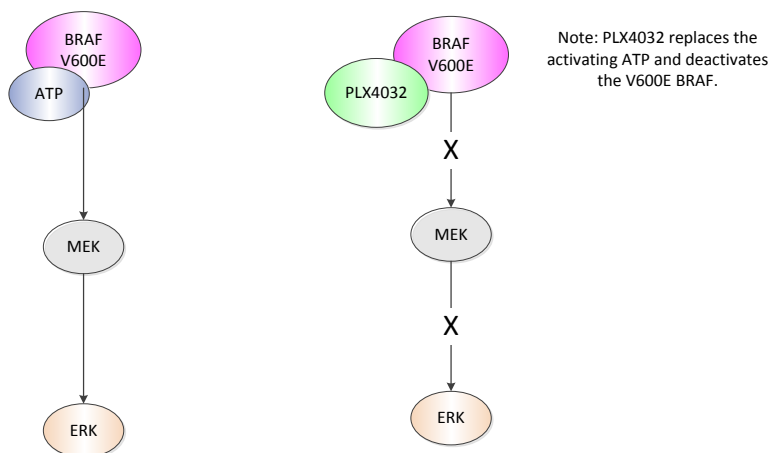
As Babchia et al state:

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.

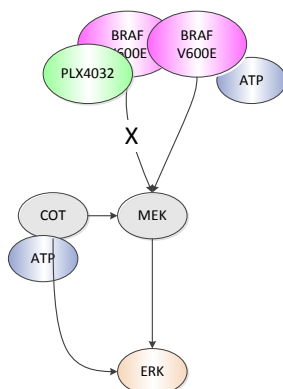
Analysis of Akt phosphorylation levels in rapamycin-treated cells showed that inhibition of mTOR greatly increased Akt phosphorylation without affecting Akt levels in uveal melanoma cell lines. This phenomenon was observed at a concentration as low as 10 nM rapamycin. ...If our

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



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. PDGFR β denotes platelet-derived growth factor receptor β , and *RAS*-GTP *RAS* in its active, GTP-bound state."

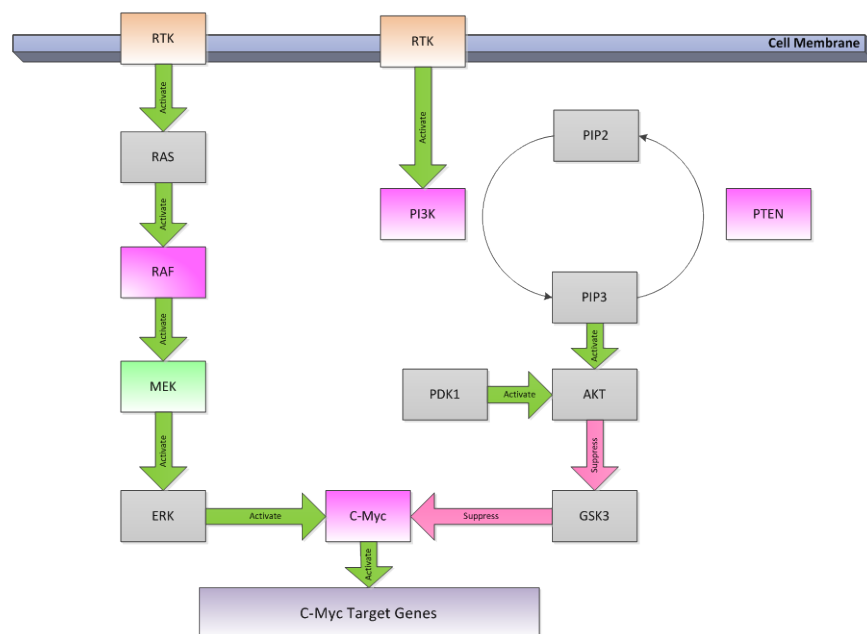
6.8.4 PTEN

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 NCBI⁶¹:

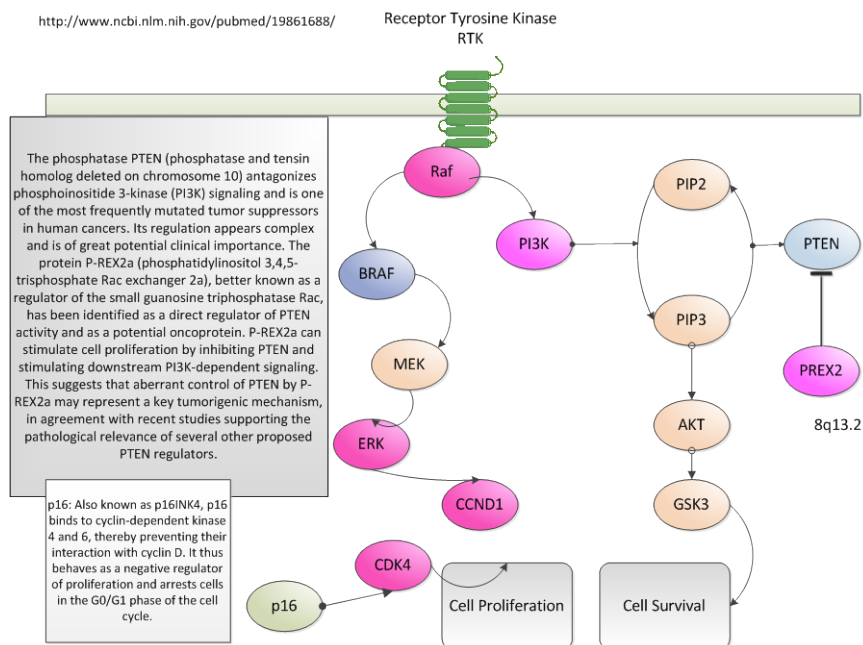
⁶¹ <http://www.ncbi.nlm.nih.gov/gene/5728>

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.

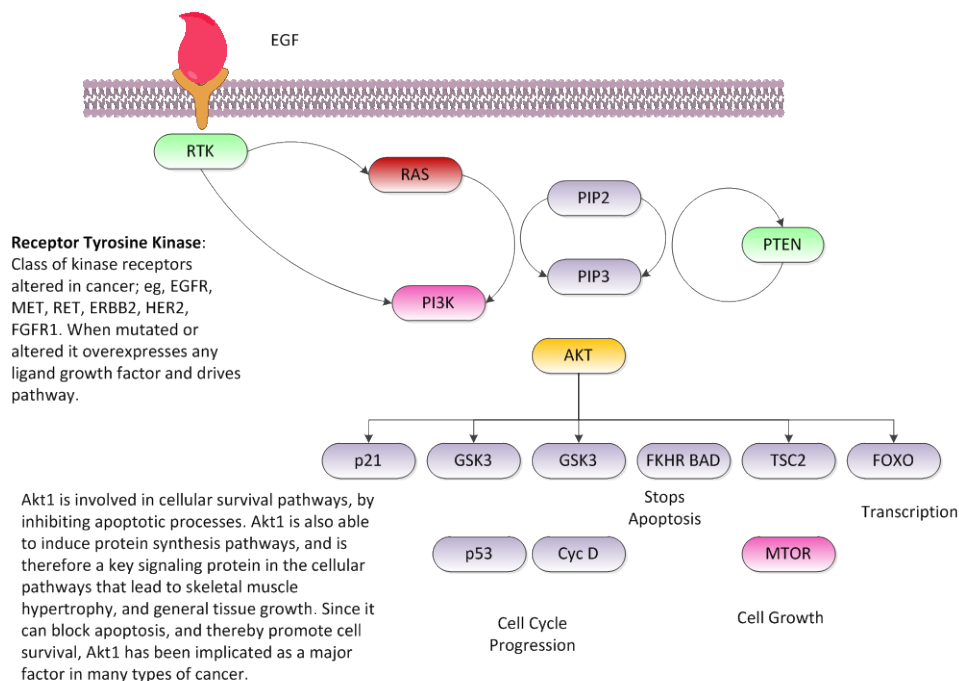
First the PTEN pathway as shown below:



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.



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 state⁶²:

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.

The authors state in McMenamin et al state:

PTEN expression in prostate tissue.

6.8.5 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 kinase or CDK. The activated cyclin-CDK complex phosphorylates 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.

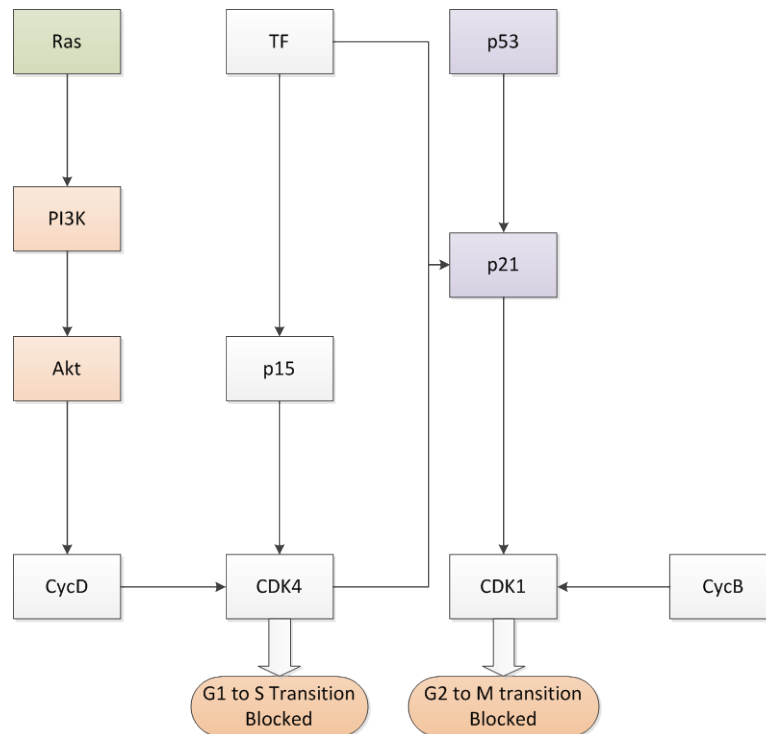
As stated in NCBI⁶³:

⁶² <http://jama.ama-assn.org/content/304/24/2744.full>

⁶³ <http://www.ncbi.nlm.nih.gov/gene/595>

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, 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:



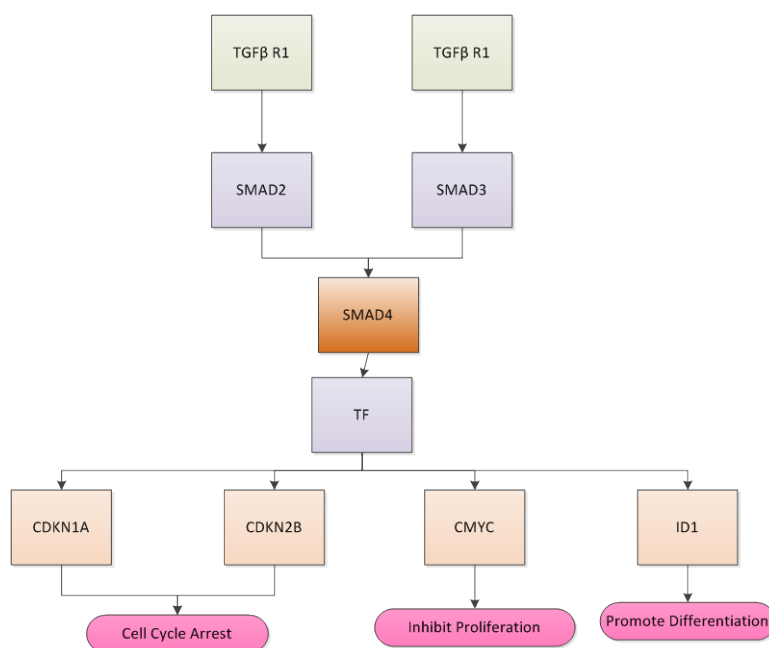
Note that Cyc D if not regulated will in turn fail to regulate the blocking of the G1 to S transition.

6.8.6 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.

| <i>Gene</i> | <i>Function</i> | <i>Disease</i> | <i>Pathway</i> |
|----------------|---------------------------|--|----------------|
| 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 NCBI⁶⁴:

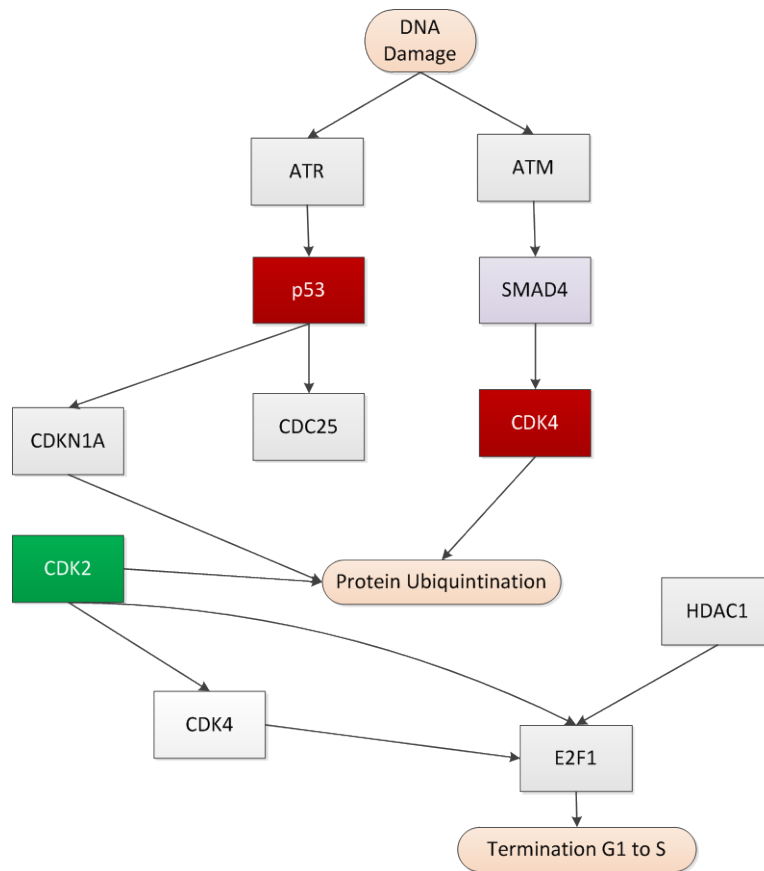
This gene encodes a member of the Smad family of signal transduction proteins. Smad proteins are phosphorylated and activated by transmembrane serine-threonine receptor kinases in response to TGF-beta signaling. The product of this gene forms homomeric complexes and heteromeric complexes with other activated 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 pathway⁶⁵:

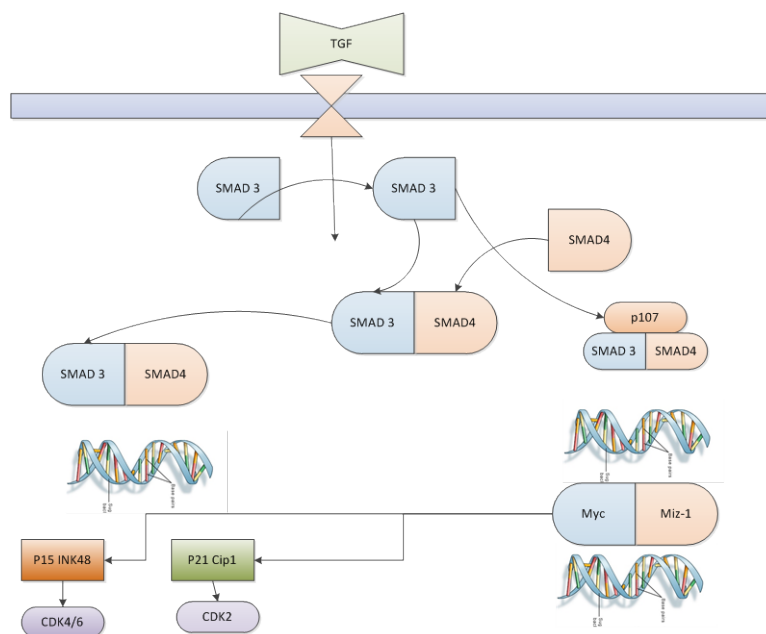
⁶⁴ <http://www.ncbi.nlm.nih.gov/gene/4089>

⁶⁵ 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.

6.8.7 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 species⁶⁶.

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.

⁶⁶ Also see <http://www.ncbi.nlm.nih.gov/gene/6696> also see <http://www.wikigenes.org/e/gene/e/6696.html>

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.

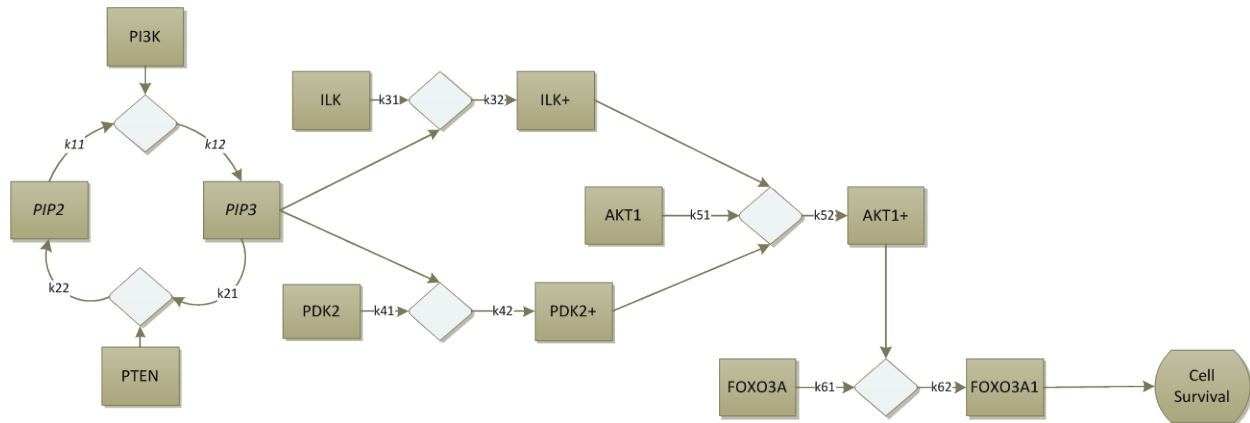
6.8.8 FOXO

The FOXO gene, specifically FOXO3a, forkedhead box zero gene, is located at 6q21 in humans and is a key nuclear transcription regulator. It has the ability to mediate cell cycle arrest, DNA repair, 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.

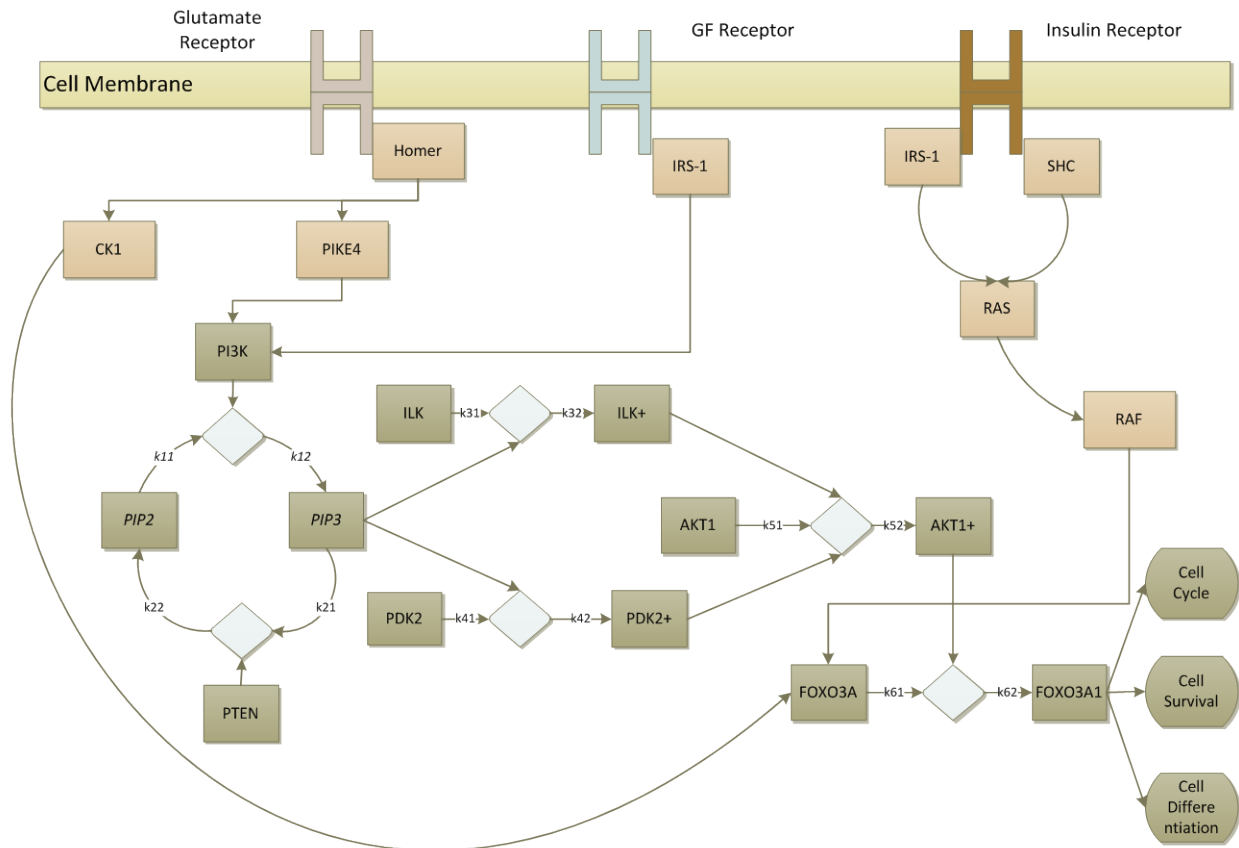
As noted by van der Heide et al, FOXO is a major player in pathways activated by Glutamate and insulin. We will depict that detail later. However the nexus to the insulin activator may also provide a connection to the role that inflammation may have in PCa and especially Type 2 Diabetes and its related 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 Essaghiri 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 ligand 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.

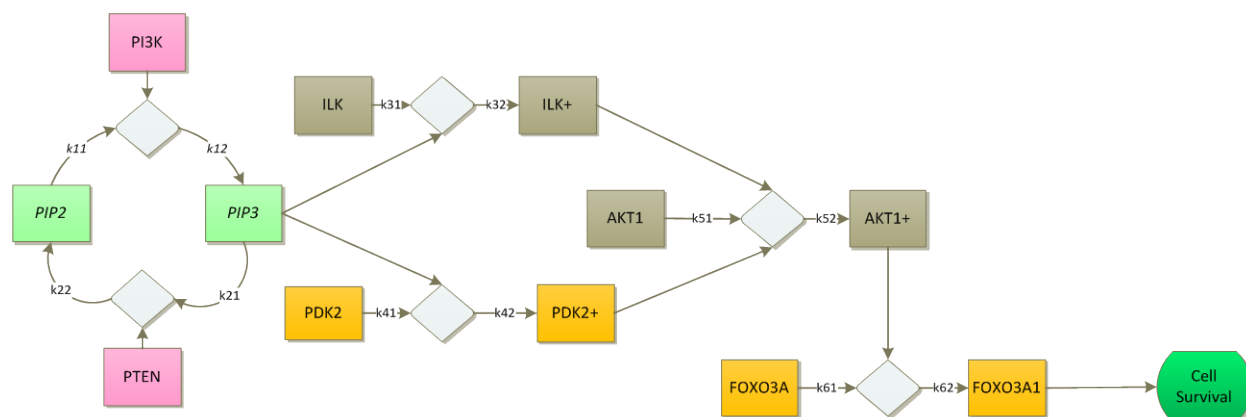
They continue:

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.

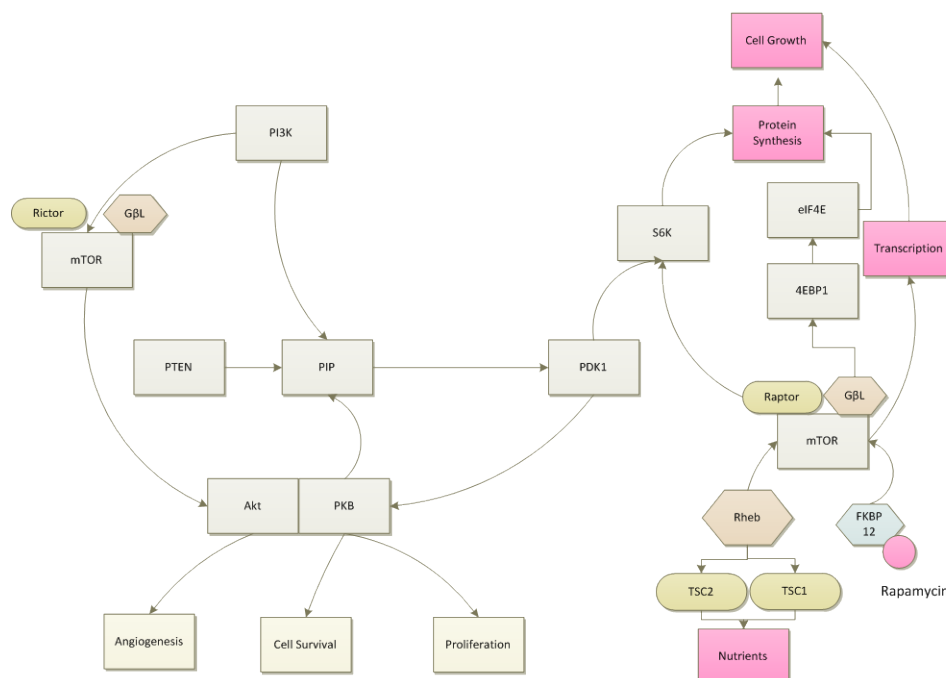
Thus FOXO control is a strategic part of controlling cell growth and stability.



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.

6.8.9 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.

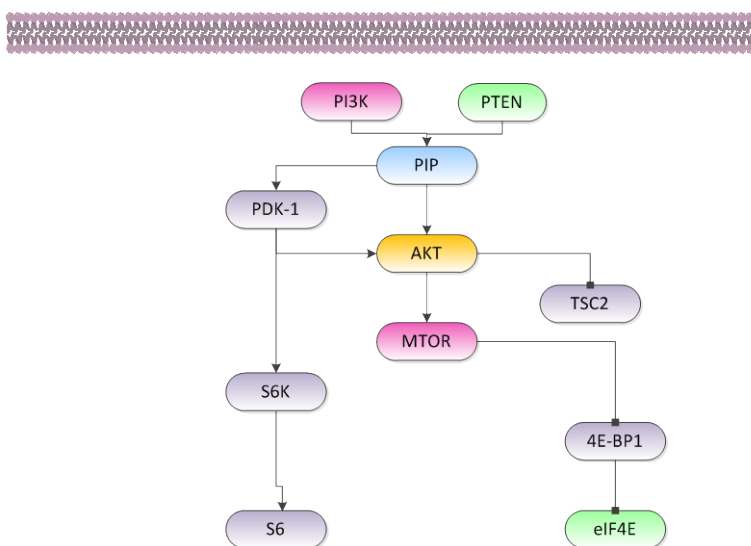
LoPiccolo et al state:

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:

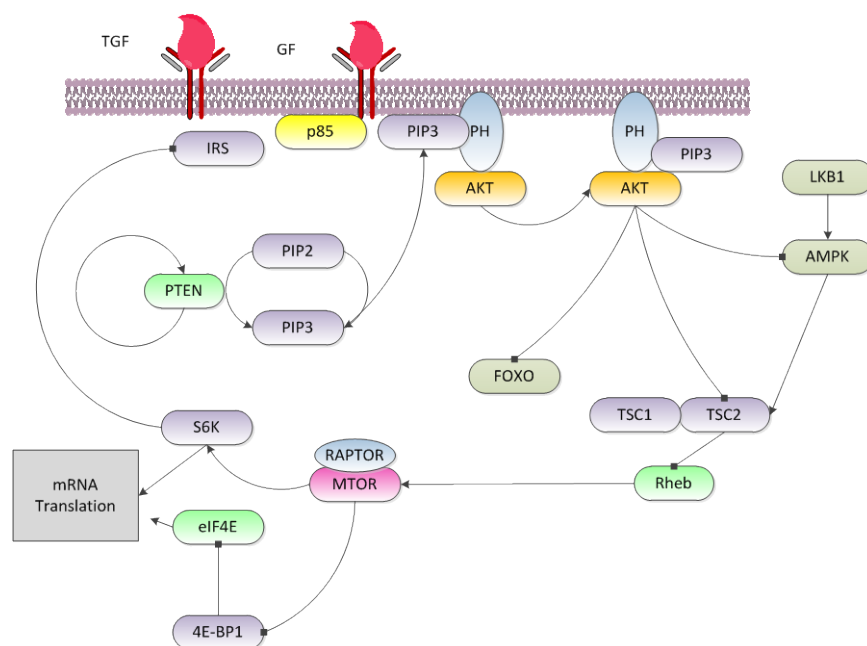


As we have shown with the more complex Weinberg model, here mTOR and PTEN play a strong role in the overall control. The authors show the points of possible control. The complexity of the pathways will be a challenge. It is less an issue of size complexity than a feedback and instability complexity. 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.

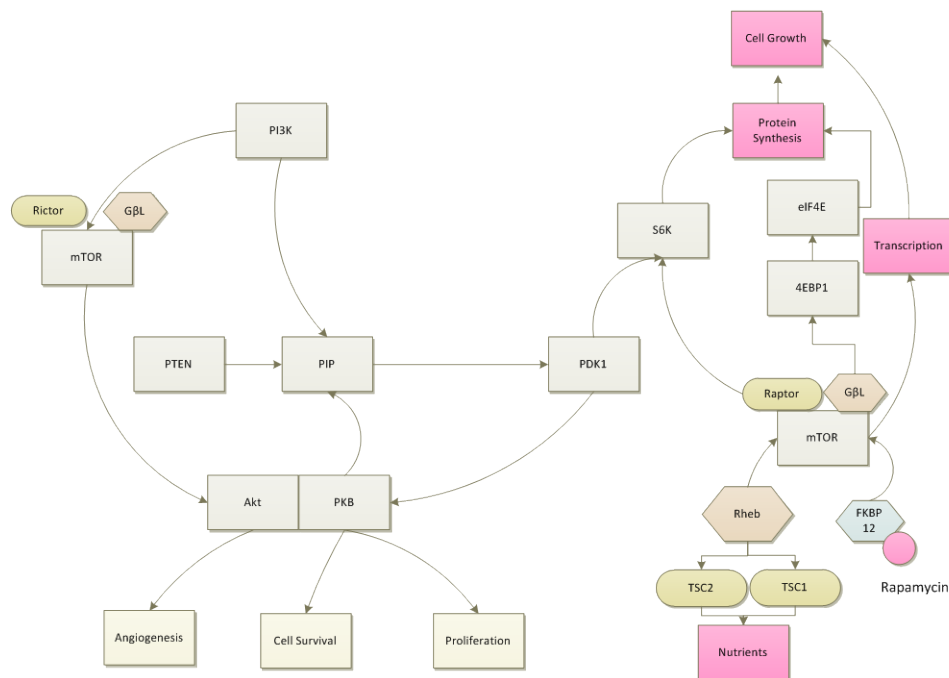
In fact this complex, believed until recently to be completely insensitive to the effect of Rapamycin, after long-term exposure to Rapamycin is able to prevent mTOR-mediated Akt phosphorylation and the activation of the mTOR pathway. Another component, the TSC1/TSC2 complex located upstream of mTOR, 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.

6.8.10 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 ($\pm p53$ 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:

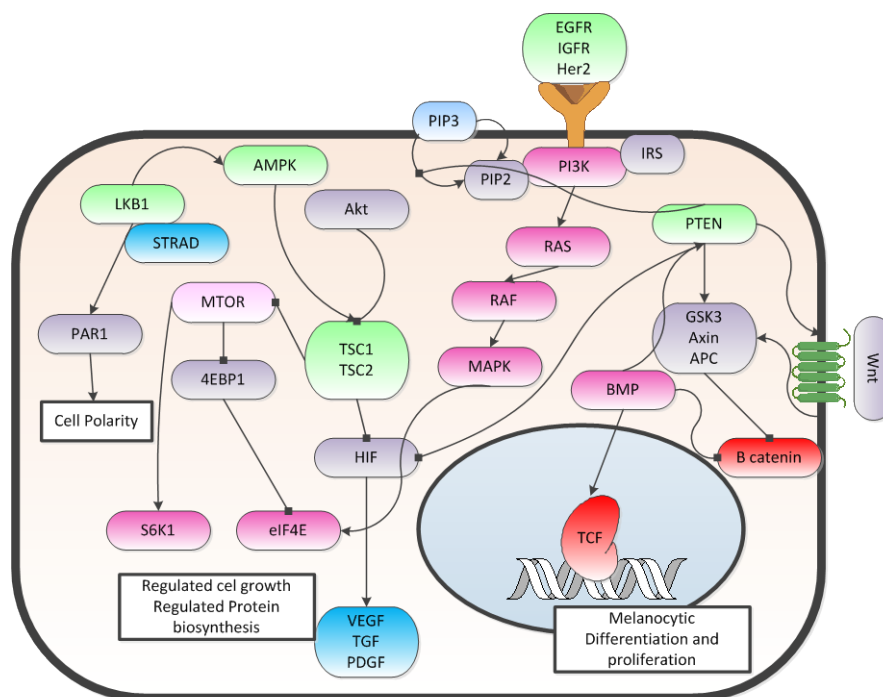
The LKB1-AMPK signaling pathway serves as a critical cellular sensor coupling energy homeostasis to cell growth, proliferation, and survival. However, how tumor cells suppress this signaling pathway to gain growth advantage under conditions of energy stress is largely unknown.

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.

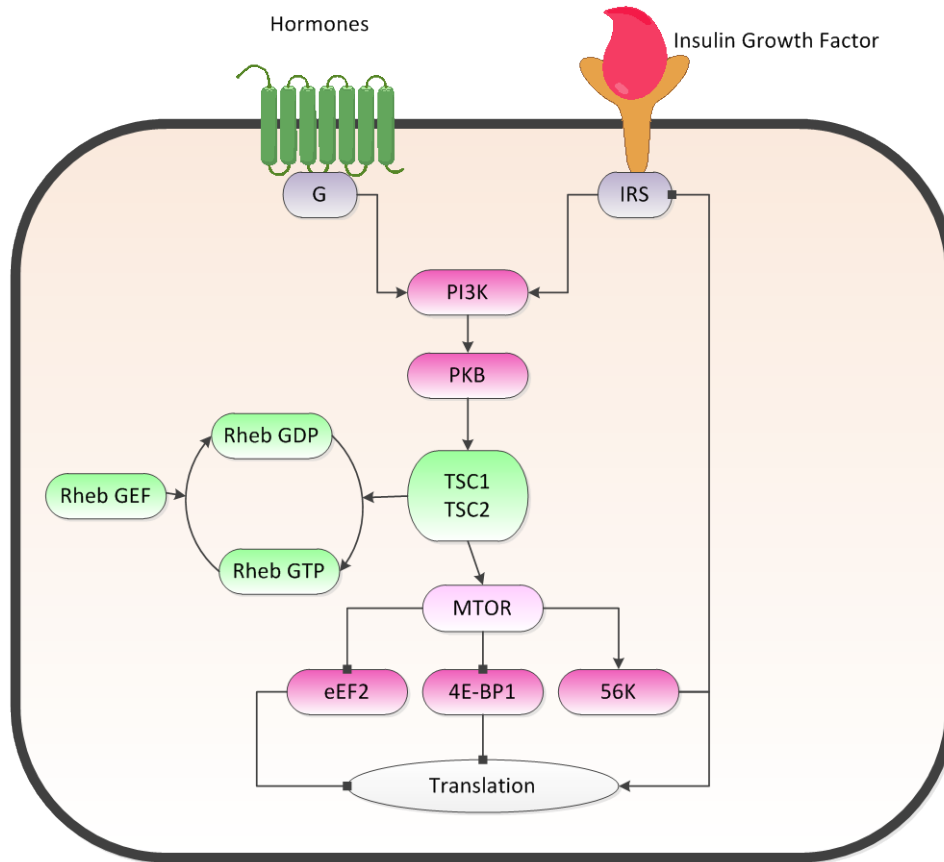


From: Bauer and Stratakis

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 growth⁶⁷. 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:

⁶⁷ 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

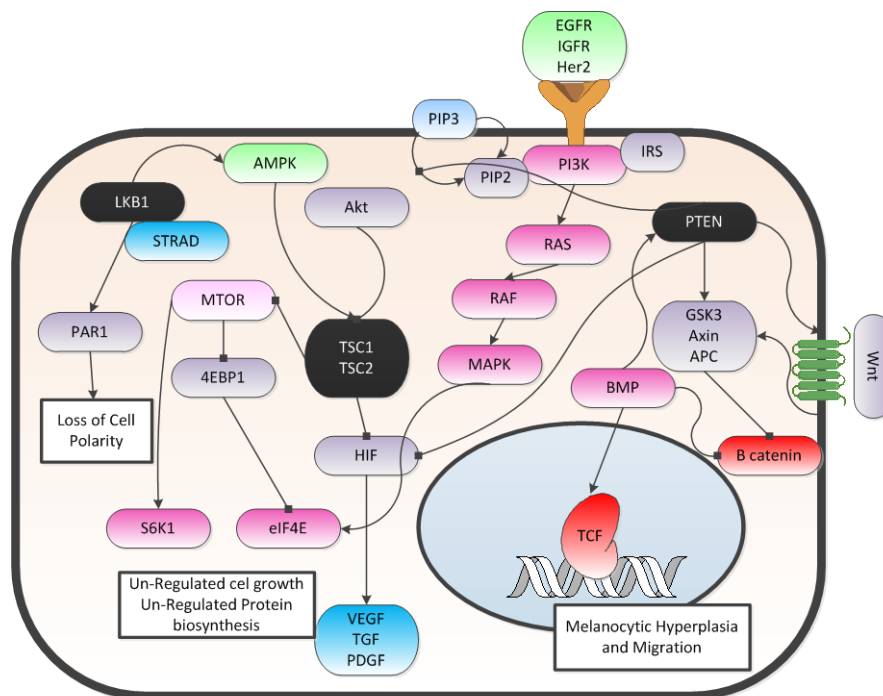
(ii) the PI3K/AKT pathway.

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 of 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 have⁶⁸:

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 transcriptional splice variants of this gene have been observed but have not been thoroughly characterized.

From the results of Shaw et al we have⁶⁹:

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.

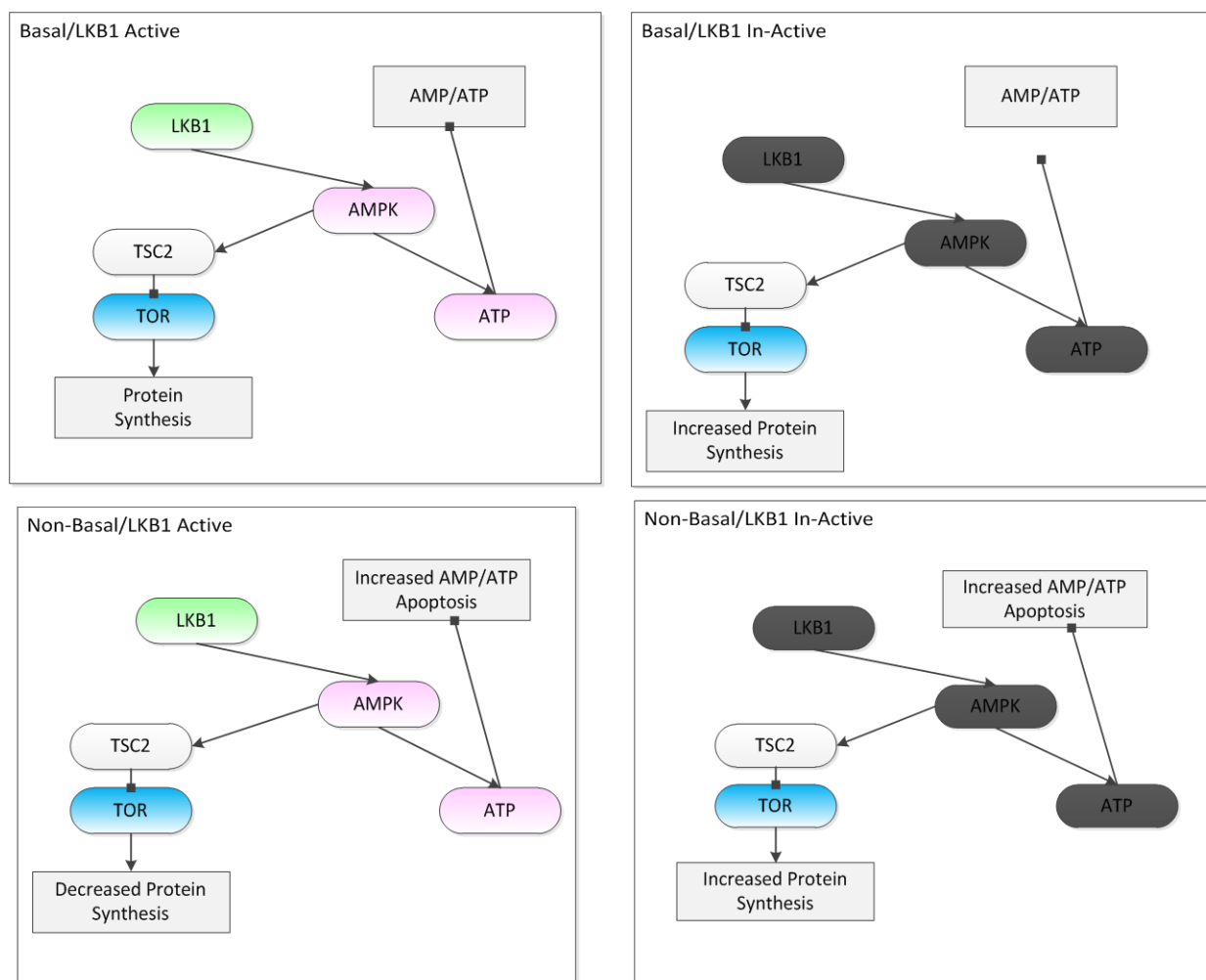
LKB1-deficient murine embryonic fibroblasts show nearly complete loss of Thr-172 phosphorylation and downstream AMPK signaling in response to a variety of stimuli that activate AMPK. Reintroduction of WT, but not kinase-dead, LKB1 into these cells restores AMPK activity. Furthermore, we show that LKB1 plays a biologically significant role in this pathway, because LKB1-deficient cells are hypersensitive to apoptosis induced by energy stress.

⁶⁸ http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene&cmd=retrieve&dopt=default&rn=1&list_uids=6794

⁶⁹ <http://www.ncbi.nlm.nih.gov/pubmed/14985505>

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:



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.

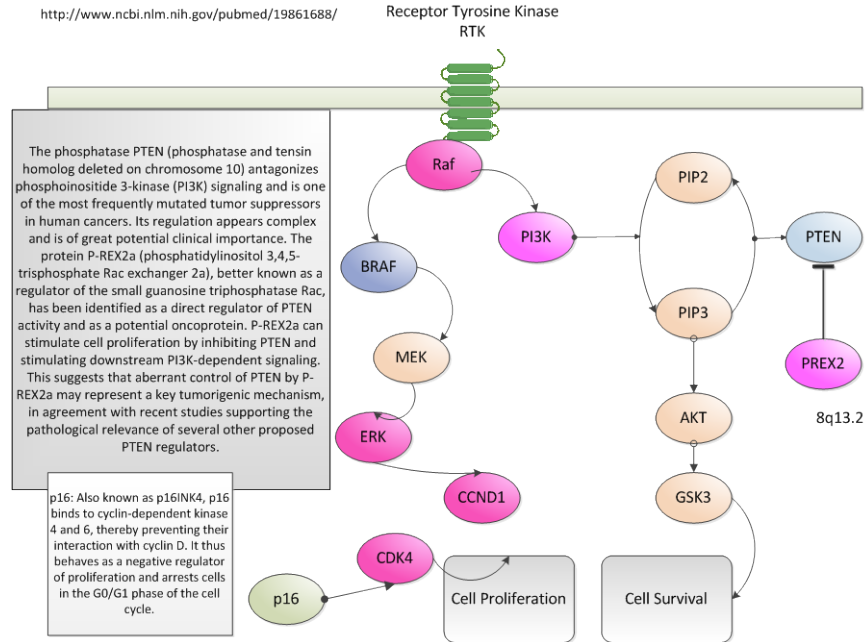
2. LKB1 is a gene related to the control from decreased nutrients. However we have the angiogenesis issue related to the increased nutrition of malignant cells. However on the counter side we have the Warburg effect as a counter to normal metabolism, namely cancer cells are anaerobic metabolic systems. 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.

6.8.11 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 seems 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 cancer—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 qRT-PCR 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:

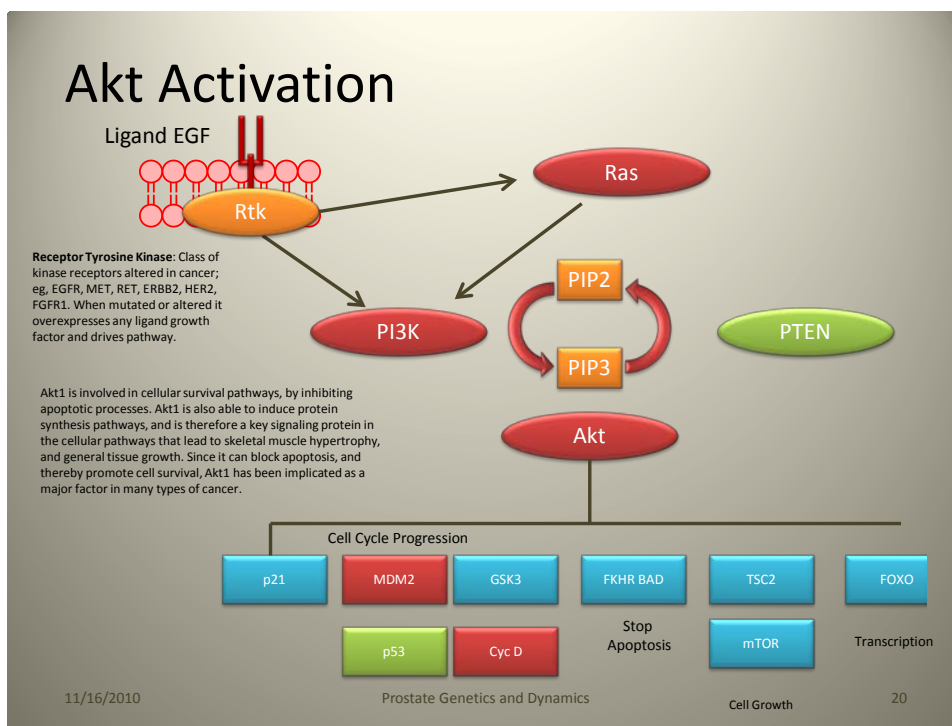
This corresponded to an average mutation rate of 30 per Mb. However, the mutation rate varied by nearly two orders of magnitude across the 25 tumours. The acral melanomas showed mutation rates comparable to other solid tumour types (3 and 14 mutations per Mb), whereas melanomas from the trunk harboured 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.

6.8.12 PTEN

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 state⁷⁰:

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.

⁷⁰ <http://jama.ama-assn.org/content/304/24/2744.full>

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.

The authors state in McMenamin et al state:

PTEN expression in prostate tissue.

A, PIN.

A1, H&E-stained section of PIN.

A2, positive staining for PTEN in PIN.

B, prostate cancer (Gleason score, 3 + 3 = 6).

B1, H&E-stained section.

B2, positive staining for PTEN in prostate cancer.

C, an example of heterogeneous expression of PTEN in a case of prostate cancer (Gleason score, 3 + 5 = 8).

C1, prostate cancer. Gleason grade 3 cancer (large black arrow), Gleason grade 5 cancer (open arrow), and PIN (short black arrow). H&E-stained section.

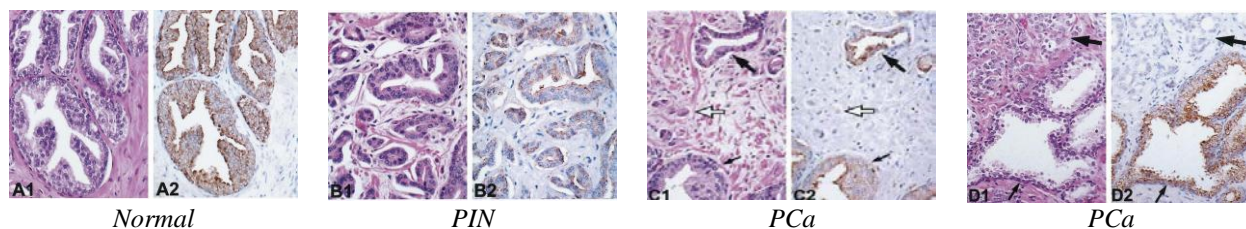
C2, Gleason grade 3 prostate cancer stains positively for PTEN (see large black arrow). In contrast, Gleason grade 5 prostatic carcinoma is negative for PTEN expression (open arrow). PIN shows positive staining for PTEN (short black arrow).

D, benign prostate acinus with surrounding prostate cancer (Gleason score, 4 + 3 = 7).

D1, H&E-stained section. Small arrow, benign prostatic glandular epithelium; large arrow, prostatic adenocarcinoma).

D2, positive staining for PTEN in benign prostate tissue and absence of staining for PTEN in prostate cancer.

Now look at A below and we see typical PIN, namely the overgrowth of the acinus with luminal cells and the closing of the lumen itself. The nucleoli structure is not show at this magnification. We can see in the PTEN stained slide the strong reflection of PTEN activity. PTEN is generally active in PIN and as is shown below it can become inactive in PCa.



Now the slide B is the PIN slide above is a lower grade PCa. Note the growth of new glandular structures and the elimination of the connective stroma. The new glands have varying shapes yet

for the most part retain some form. The PTEN expression is shown on the right and it demonstrates that the cells still express PTEN.

The following in C is PTEN expression in ever increasing Gleason levels of PCa. In C we can see on the slide 1 the clusters of small PCa cells and then when stained for PTEN the large clusters exhibit PTEN but the small ones no longer do. Somewhere they have lost PTEN expression capability. The exact mechanism of this loss does not yet seem to be apparent. We see the same in slide D below. PTEN expressed in PCa clusters which have form and then none in the more sporadic clusters.

In the Doctoral thesis by Hermans he states:

PTEN is most frequently inactivated in several sporadic human cancers including, glioblastomas, endometrial cancer, and prostate cancer, either by mutation, homozygous deletion or promoter methylation.

Loss of one PTEN allele without inactivation of the second allele is also found in prostate cancer. This suggests that PTEN haplo-insufficiency plays a role in tumorigenesis. Loss of PTEN is detected, in 39-68% of primary prostate cancer samples and in 23% of PIN lesions. Loss of one copy of the 10q region, where PTEN maps, might also be explained by the presence of a second tumor suppressor gene located nearby PTEN.

We have screened 14 genes mapping in a ~3 Mbp region around PTEN for mRNA expression alterations in prostate cancer xenografts and cell lines. The frequency of complete PTEN inactivation reported in clinical prostate cancer is highly variable. This might partially be due to the different techniques used to detect complete inactivation of PTEN and to the different stages of disease analyzed.

The major mechanism of complete PTEN inactivation is by homozygous deletion. Most common point mutations produce a premature stop codon. Other frequent mutations are point mutations in the active site of the phosphatase domain of PTEN. Complete inactivation of PTEN is most often detected in metastatic prostate cancer, up to 60%, and less frequently in primary tumours, in ~15%. In prostate cancer PTEN is the most frequently mutated tumor suppressor gene found to date.

6.8.13 EGFR, ERBB and Neu

From the paper of Deutsch et al (2004) we have:

ERBB2 is a receptor tyrosine kinase that belongs to the epidermal growth-factor receptor family. Overexpression of ERBB2, which is seen in 20–30% of breast and ovarian cancers, is the result of gene amplification and is associated with a poor prognosis. In prostate cancer, the assessment of ERBB2 overexpression has been more difficult (procedural differences, and variability of tissue fixation protocols). Overexpression of ERBB2 has been associated with progression to androgen-independence in prostate cancer cells.

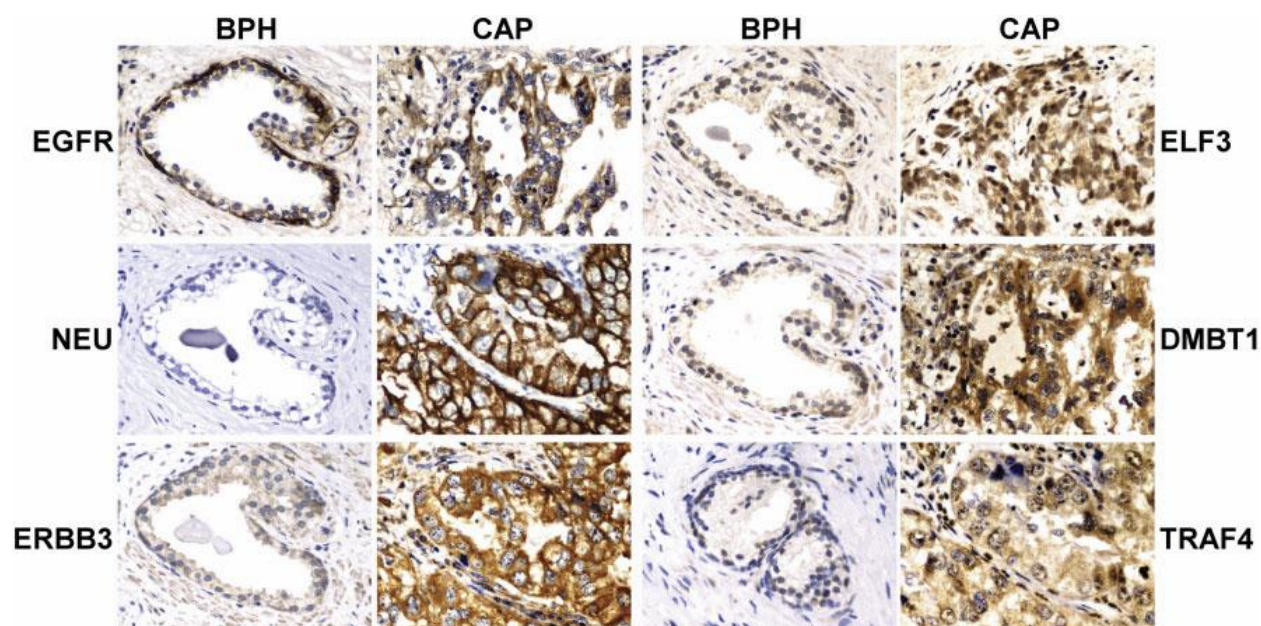
First, as discovered in SCID mice with the prostate cancer xenograft LAPC (Los Angeles prostate cancer), *ERBB2* is overexpressed in androgen-independent cells. Second, overexpression of *ERBB2* in androgen-dependent prostate-cancer cells activates the AR in a ligand independent fashion, conferring androgen-independent growth on these cells. FISH studies of primary prostate cancer samples suggest that *ERBB2* amplification and *ERBB2* overexpression are significantly correlated with DNA content, advanced stage, and high-grade lesions.

The *PI3K* pathway is one of the most important signalling pathways activated by receptor tyrosine kinase. The *PI3K* pathway controls several important biological functions, such as cell growth regulation, apoptosis, and migration. As well as androgen signalling, which plays an essential part in the survival of prostate-cancer cells, the *PI3K* pathway is another important survival signal for these cells.⁵² The androgen-signalling and *PI3K* pathways can compensate for each other in regulating the growth of prostate cancer cells. Since androgen treatment can rescue cells from apoptosis induced by *PI3K* inhibitors, these data suggest that the pathways are interconnected.

Furthermore, the *PI3K* pathway interferes with AR-mediated prostate-cancer cell survival and could change the stability of the AR protein. In addition, the IGF1 receptor can activate the *PI3K* pathway, resulting in phosphorylation of AR. The enhancement of AR transcriptional activity in the presence of epidermal growth factor (EGF) or IGF1 shows the synergistic interactions between these pathways.

Conversely, androgens promote overexpression of the EGF receptor, which is essential for androgen-induced proliferation and survival.

In a paper by Li et al they demonstrate the expression of EGFR, NEU and ERBB. We reproduce below the results from the Li et al paper:



6.8.14 AR

The Androgen Receptor, AR, is located on Xq12. Androgens mediate a wide range of developmental and physiological responses and are especially important in male sexual differentiation and pubertal sexual maturation, the maintenance of spermatogenesis, and male gonadotropin regulation. The principle steroidal androgens, testosterone and its metabolite DHT (5-Alpha-Dihydrotestosterone), mediate their biological effects predominantly through binding to the AR (Androgen Receptor), an androgen-inducible member of the nuclear receptor super-family of transcription factors.

The normal function of the Androgen Receptor is as follows:

1. Testosterone enters the cell
2. If 5- α -Reductase is present the testosterone is converted to dihydrotestosterone, DHT.
3. The DHT then binds with the AR and the entity undergoes a transformation and releases heat shock proteins, HSPS
4. Then there is a phosphorylation
5. The AR translocates to the nucleus where it dimerizes, and there is DNA binding.
6. Target genes are then transcribed.

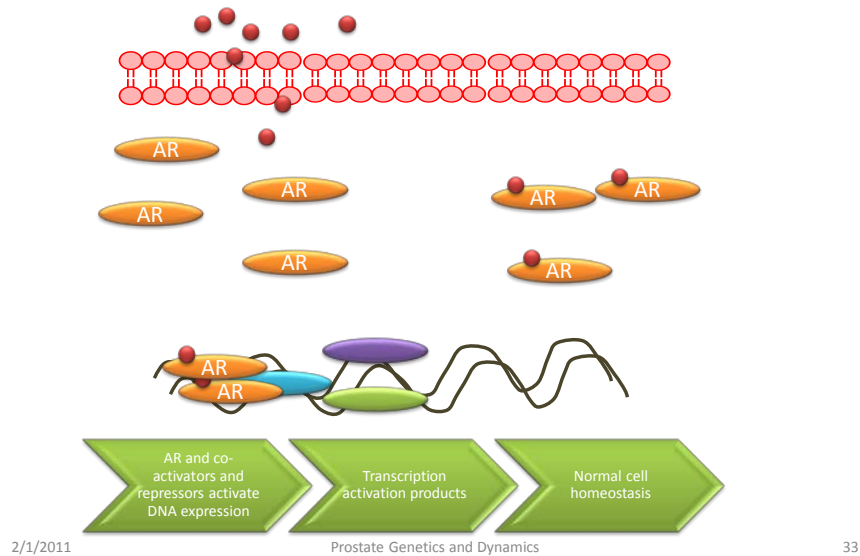
AR mediates transcription of proteins which are essential for normal development. However as PCa progresses there is at first normal AR operation, then it is enhanced, and then the PCa which was dependent upon the AR function can become independent of it altogether. We depict that process below.

AR Process



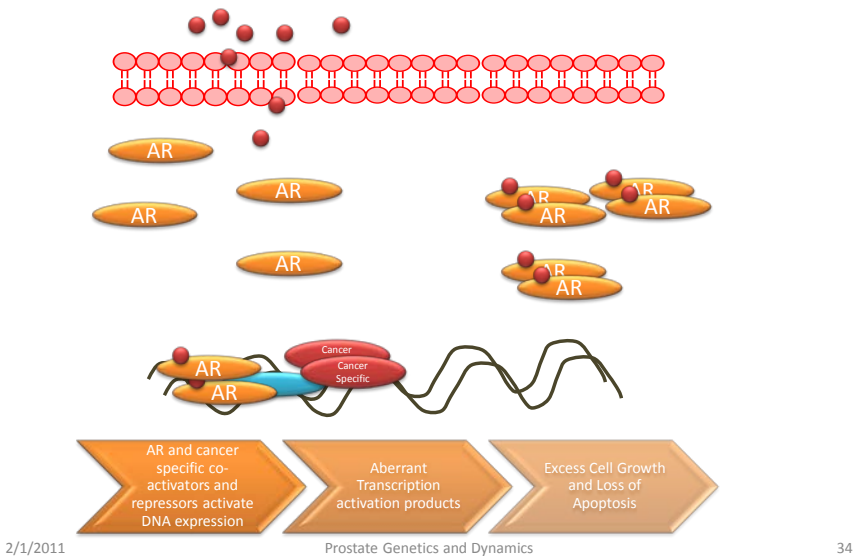
In normal AR operations, we show below the Testosterone coming into the cell and then it binds with the AR. It is this normal bonding which gives the AR the ability to manage a significant portion of the normal growth of the prostate cell. We use the graphics from Turner (2010) as modified below:

Normal AR Operations



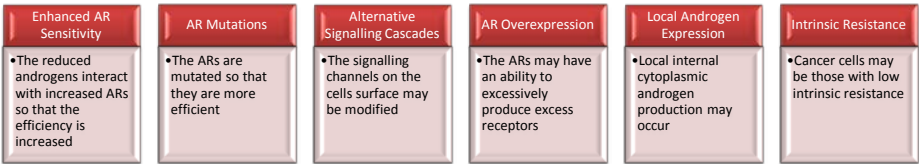
In the case of PCa we see the AR playing the role of excess growth enhancer.

Cancer and AR Operations



As is best understood, the progression towards AR resistant PCa follows the path shown below.

Hormone Refractory Options



The diagram illustrates the molecular mechanisms of prostate cancer progression, showing pathways from androgen resistance to cell growth and apoptosis.

Top Section: Androgen Receptor (AR) and Signaling

- Alternative Signalling Cascade:** A green oval representing an alternative signaling pathway.
- Enhanced Sensitivity:** A cluster of orange ARs (Androgen Receptors) with red dots (ligands) bound to them.
- AR Mutations:** A cluster of purple ARs (Androgen Receptors) with red dots (ligands) bound to them.
- AR Overexpression:** A cluster of orange ARs (Androgen Receptors) with red dots (ligands) bound to them.
- Local androgen production:** A cluster of orange ARs (Androgen Receptors) with red dots (ligands) bound to them.

Bottom Section: Androgen Resistance and Cancer Progression

- Intrinsic Resistance:** A cluster of orange ARs (Androgen Receptors) with red dots (ligands) bound to them.
- AR and cancer specific co-activators and repressors activate DNA expression:** A cluster of orange ARs (Androgen Receptors) with red dots (ligands) bound to them.
- Aberrant Transcription activation products:** A cluster of orange ARs (Androgen Receptors) with red dots (ligands) bound to them.
- Excess Cell Growth and Loss of Apoptosis:** A cluster of orange ARs (Androgen Receptors) with red dots (ligands) bound to them.

Prostate Genetics and Dynamics

The androgen receptor (AR), when complexed with 5-dihydrotestosterone (DHT), supports the survival and proliferation of prostate cells, a process critical for normal development, benign prostatic hypertrophy, and tumorigenesis. However, the androgen-responsive genetic pathways that control prostate cell division and differentiation are largely unknown.

*Notably AR represses the expression of several key cell cycle inhibitors, while modulating members of the **wnt and notch signaling pathways**, multiple growth factors, and peptide hormone signaling systems, and genes involved in MAP kinase and calcium signaling. Analysis of these data also suggested that **p53 activity is negatively** regulated by AR activation even though p53 RNA was unchanged. Experiments in LNCaP prostate cancer cells reveal that AR **inhibits p53 protein accumulation** in the nucleus, providing a post-transcriptional mechanism*

*by which androgens control prostate cell growth and survival. In summary these data provide a comprehensive view of the earliest events in AR-mediated prostate cell proliferation *in vivo*, and suggest that nuclear exclusion of p53 is a critical step in prostate growth.*

The authors continue:

AR induces cell proliferation and apoptosis in part because of its effects on cell-cell communication, particularly the stromal-epithelial interaction . As expected, the insulin-like growth factor (IGF-1) signaling system, which plays an essential role in prostate growth, was regulated at the level of ligand (IGF-1 was induced), extracellular binding protein (IGF-BP3 was repressed), and receptor (the IGF-1 receptor-1 exhibited biphasic expression). Also as expected, epidermal growth factor, which is induced by androgens in the prostate epithelium was upregulated . In addition to these well studied factors, several genes with potentially novel roles in the prostate were identified .

These include the transforming growth factor-2 (TGF-2) secretory partner latent TGF-binding protein- 1 (Ltbp1), which was repressed. Although the role of TGF-proteins in growth repression has been documented (e.g. Ref. 44), latent TGF-binding protein 1 function in the prostate has not been extensively studied, although its expression might be frequently reduced in PCa. DHT also repressed granulin/epithelin (Grn), a cysteine-rich growth factor expressed throughout the reproductive tract that regulates growth in multiple epithelial cell types.

Given the role of Grn in certain epithelial neoplasias, it would be interesting to examine its expression in PCa. Finally, DHT led to the down-regulation of ephrin-A1/B61 (Efna1), a ligand for the Eph receptors expressed in various epithelia. EFNA1 regulates cell growth and inhibits tumor angiogenesis; its function in the prostate is unknown.

In short, other than p53 suppression, and a collection of other genes, there is not significant addition to what is already known. The Chen and Sawyers discussion ends with:

*Despite the fact that AR occupies such a central role in prostate physiology and pathology, there is **little insight into the direct AR target genes responsible for disease progression**. One very intriguing possibility, based on the high frequency of TMPRSS2-ETS fusion, is that the primary effect of antiandrogen therapy is to reduce expression of this presumed oncogene. Even though AR is overexpressed in more advanced stages of prostate cancer, recent profiling studies indicate that many AR target genes are actually expressed at lower levels in high-grade and metastatic lesions.*

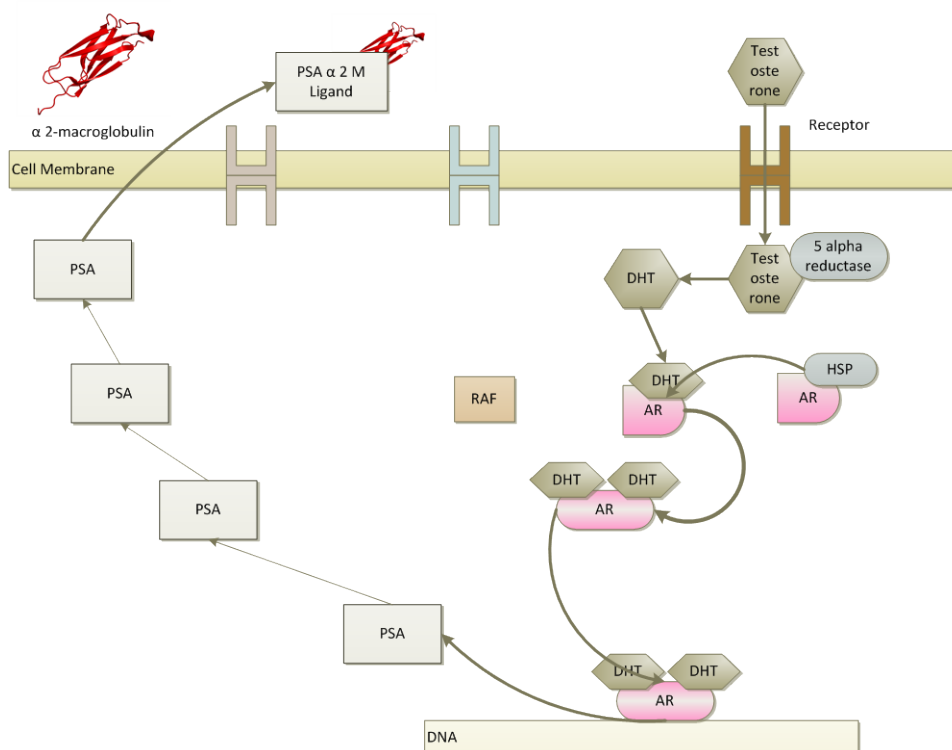
This finding is consistent with older data that cancers with a high Gleason grade often produce lower levels of PSA and other markers of differentiation. Therefore, the relationship between the AR pathway and castrate resistance may also reflect the differentiation state of late-stage tumors. A small subset of very aggressive prostate cancers (small cell variant) does not express AR.

Clearly the importance of the AR is critical in PCa as it progresses and yet as noted above the full pathway development is still lacking.

6.8.15 GRP78

In the normal course of prostate cell homeostasis, PSA is produced by the binding of Testosterone to the ligand on the luminal cell and the resulting transformation to DHT and its binding to the Androgen Receptor which acts in turn as a transcription factor for PSA. PSA exists mostly in complexed and inactive form; bound to alpha 2 macroglobulin and alpha 1 antichymotrypsin. 40% of the bound is with alpha 2 macroglobulin. Half-life PSA is 2.2-3.2 days. The baseline may takes weeks to return to normal after certain procedures.

We show this process below (See Feldman and Feldman, 2001):



In the above the PSA is released and then is free, it may bind with alpha 2 macroglobulin or with alpha 1 antichymotrypsin. In a benign configuration there is generally 35% of the PSA remaining free and unbound.

Now in a recent paper by Misra et al (2011) they discuss the impact of GRP78 in cancerous cells. In a recent write-up of the work the authors state⁷¹:

Using human prostate cancer cells in a laboratory culture, the team found that an antibody reacts with a cell surface receptor called GRP78 on the cancer cells to produce more PSA. The

⁷¹ http://www.eurekalert.org/pub_releases/2011-01/dumc-rlw011311.php

PSA arises inside of the cancer cell and then moves outside of the cell, where it can bind with the same antibody, called alpha2-macroglobulin (α 2M).

The PSA forms a complex with the antibody that also binds to the GRP78 receptor, and that activates several key pathways which stimulate cancer cell growth and cell movement and block cell death.

The study bolsters the case for measuring PSA as a marker of tumor progression, as well as for monitoring for α 2M antibody levels.

That is the release of GRP78 enforces the release of PSA, bound to alpha-2-M which increases PSA total and reduces PSA free. The GRP78 is then an added marker for excess cell growth, and thus a putative marker for PCa and putatively PSA total and percent free than has substantially increased significance for monitoring PCa.

Also this is a positive feedback loop, the link to the enhancement of Akt then is an enhancement of cell proliferation and growth even with the negative influence of PTEN if present.

GRP78, a well characterized chaperone in the endoplasmic reticulum, is critical to the unfolded protein response. As Lee (2007) states:

The glucose-regulated protein GRP78, also referred to as BiP (immunoglobulin heavy-chain binding protein), was discovered in the late 1970s together with GRP94 and GRP58 as cellular proteins induced by glucose starvation. Residing primarily in the ER, GRP78 belongs to the HSP70 protein family, which plays critical roles in the stress of oncogenesis.

In addition to facilitating proper protein folding, preventing intermediates from aggregating, and targeting misfolded protein for proteasome degradation, GRP78 also binds Ca_{2+} and serves as an ER stress signaling regulator. In nonstressed cells, GRP78 binds to ER transmembrane sensor proteins PERK, IRE1, and ATF6 and maintains them in an inactive form. When unfolded proteins pull GRP78 away from them, these pathways are activated, sending signals to the nucleus to trigger the UPR.

As Pootrakul et al state:

The glucose-regulated proteins (GRP) were initially identified as such in transformed chick embryo fibroblasts growing in glucose-deprived medium. The most well studied member of the GRP family is Grp78, a 78-kDa protein also recognized as immunoglobulin heavy-chain binding protein (BiP).

Normal functions of Grp78, which resides in the endoplasmic reticulum (ER) lumen, include proper folding and assembly of other polypeptides leading to formation of functional proteins, retention of unassembled precursors to the ER, targeting mis-folded protein for degradation, ER Ca_{2+} binding, and the regulation of trans-membrane ER stress inducers. The involvement of Grp78 in enhanced cell survival is suggested by the remarkable elevation of GRP78 transcription rates under various stress conditions.

Recently, Grp78 has been shown to directly interact with intermediates of the apoptotic pathway, blocking caspase activation, where Grp78 induction results in increased cell survival and inhibition of apoptosis.

More recently, it has been identified on the cell surface, where it has many roles. On cancer cells, it functions as a signaling receptor coupled to proliferative-antiapoptotic and promigratory mechanisms.

In the current study, Misra et al (2011) demonstrate that:

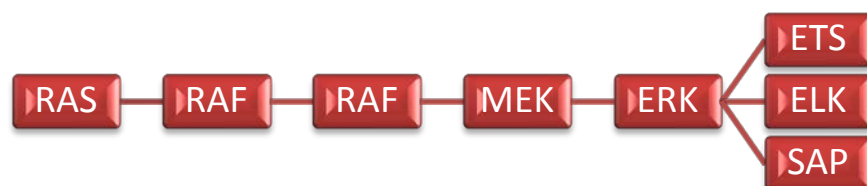
1. Ligation of prostate cancer cell surface GRP78 by its natural ligand, activated α_2 -macroglobulin (α_2 M*),
2. Results in a 2–3-fold up-regulation in the synthesis of prostate-specific antigen (PSA).
3. The PSA is secreted into the medium as an active proteinase, where it binds to native α_2 M.
4. The resultant α_2 M-PSA complexes bind to GRP78, causing a 1.5–2-fold increase in the activation of MEK1/2, ERK1/2, S6K, and Akt,
5. Which is coupled with a 2–3-fold increase in DNA and protein synthesis.

PSA is a marker for the progression of prostate cancer, but its mechanistic role in the disease is unclear. The present studies suggest that PSA may be involved in a signal transduction-dependent feedback loop, whereby it promotes a more aggressive behavior by human prostate cancer cells. We demonstrate this below:

| | 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 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.

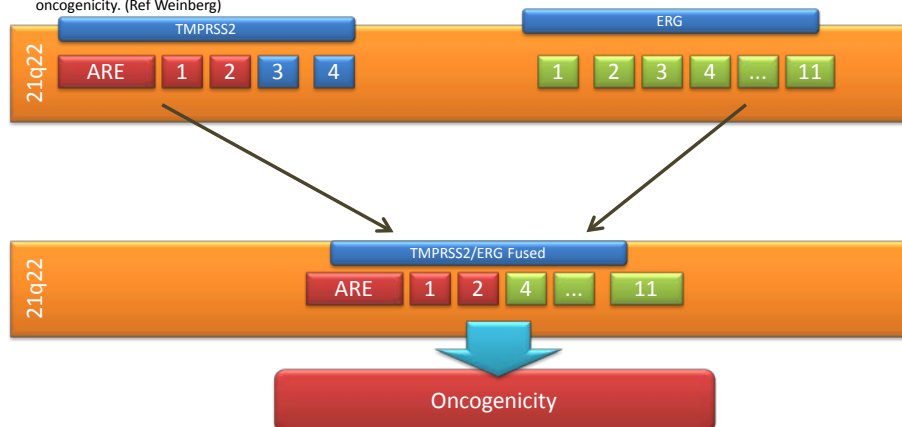


6.1.4.6 *TMPRSS2 and ERG Fusion*

The fusion of *TMPRSS* and *ERG* is another genetic promoter of PCa and it is the primary translocation gene seen.

TMPRSS2-ERG Fusion

In the case of the *TMPRSS-ERG fusion*, both genes are located on 21q22, and the fusion frequently occurs because of an interstitial deletion. The resultant fusion transcripts are androgen responsive and usually encode an ETS gene (*ERG*) truncated at its N terminus without any coding elements from *TMPRSS2*. It is unknown if the biologic consequences of misexpression of the truncated ETS family protein are different from expression of the full length protein and whether truncation contributes to oncogenicity. (Ref Weinberg)

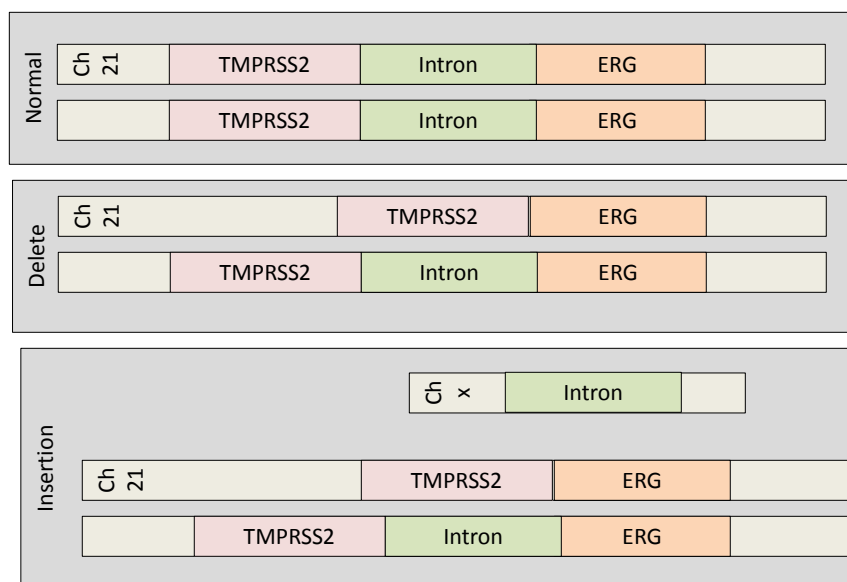


1/2/2011

Prostate Genetics and Dynamics

29

Tomlins et al discuss the various conjectures regarding the fusions. The graphic below is based upon Tomlins et al. The example below shows the normal state on 21 and then the deletion, the intron is just removed, and then an insertion where the intron is removed but inserted elsewhere. See also the work by Mani et al (2009) regarding the gene fusions in general as applied to PCa. Also the work by Demichelis et al (2009), Marucci et al (2007) Iljin et al (2006) and Esgueva et al (2010) for extensions of this description.



It is thus the fused gene that cause the problem acting as an oncogene. This is unlike the other processes, for here we actually have genetic changes in location. The intron is 3Mb long so it is a nontrivial deletion. Unlike a methylation of a base pair element this requires substantial genetic change.

As the work of King et al state:

These data suggest that TMPRSS2-ERG is insufficient to initiate prostate neoplasia and that cooperating oncogenic lesions are required. Two relatively common abnormalities in human prostate cancer are PTEN loss and MYC amplification, both of which have pathogenic roles in genetically engineered mouse models

In a 2005 paper by Tomlins et al the authors discuss the fusion of the two genes, TMPRSS2 and ERG and the prevalence of this fusion in PCa. They relate the translocation and fusion of the genes in CML where BCR-ABL is fused to create a new gene, with an associated translocation, and then discuss the juxtapositioning of promoter and enhancers of one gene being juxtaposed to a proto-oncogene. Using a technique calls Cancer Outlier Profile Analysis, COPA, they had managed to isolate the fused product of TMPRSS2 and ERG in PCa. This is a fusion on 21q22. See also the work by Rubin and Chinnaiyan (2006) on the COPA analysis.

In the work of Esgueva et al the authors indicate that this fusion has several distinct features:

1. Murine models with overexpressed ERG with and without PTEN loss show a neoplastic phenotype.

2. ERG and histological features have been correlated. This is detailed in the paper by Mosquera et al (2007).
3. Specific pathways have been shown to have been rearranged, especially estrogen signalling.
4. Somatic copy number alterations have been found to be increased in ERG enhance PCa.
5. ERG rearranged PCa have highly negative outcomes.
6. ERG rearranged response to abiraterone is different.

The conclusion that Esgueva et al then reach is that ERG rearrange PCa is a different clinical class.

6.1.4.7 *TMPRSS2:ERG HGPIN*

There have been several studies on the relationship of this fusion to HGPIN. In Mosquera et al they state:

Given the more aggressive nature of TMPRSS2-ERG prostate cancer, the findings of this study raise the possibility that gene fusion-positive HGPIN lesions are harbingers of more aggressive disease. To date, pathologic, molecular, and clinical variables do not help stratify which men with HGPIN are at increased risk for a cancer diagnosis. Our results suggest that the detection of isolated TMPRSS2-ERG fusion HGPIN would improve the positive predictive value of finding TMPRSS2-ERG fusion prostate cancer in subsequent biopsies.

The authors then continue regarding HGPIN:

In the United States, approximately 1,300,000 prostate biopsies were done in 2006 with the detection of 234,460 new cases of prostate cancer. The incidence of isolated high-grade prostatic intraepithelial neoplasia (HGPIN) without carcinoma ranges from <1% to 16% , and the risk of finding carcinoma on subsequent biopsies is 10% to 39% [median risk of 24% (6)] depending on the time of repeat biopsy and number of cores.

A decline in the predictive value of HGPIN for prostate cancer to 20% in contemporary needle biopsies is most likely due to extended biopsy techniques that yield higher rates of cancer detection. Both HGPIN and prostate adenocarcinoma share molecular anomalies, including telomere shortening, RAR hypermethylation, allelic imbalances, and several chromosomal anomalies and c-myc amplification. Overexpression of p16, ..., and altered proliferation and apoptosis in HGPIN and prostate cancer have also been shown...In particular, the TMPRSS2-ERG gene fusion prostate cancer is associated with higher tumor stage and tumor-specific death or metastasis . Two recent studies have shown the presence of TMPRSS2-ERG gene fusion in 20% of HGPIN lesions...

In a detailed study of murine models, Zong et al have concluded further the following:

1. *ERG Overexpression in Adult Murine Prostate Cells Results in Epithelial Hyperplasia and Focal PIN Lesions.*
2. *ERG-Transduced Prostate Glands Display a Skewed Cell Lineage Composition with Loss of Cytokeratin 5 (CK5)-Positive Basal Cells and Increased CD49f Expression in Luminal Cells.*
3. *ERG Overexpression Induces Up-Regulation of c-Myc and c-Jun Protein in Primary Prostate Epithelia.*
4. *Combined ERG Overexpression and p53 Deletion in Prostate Epithelia Does Not Result in Invasive Adenocarcinoma.*
5. *ERG Collaborates with Aberrant PI3K Pathway to Promote PCa Progression. Deletion of the tumor suppressor PTEN occurs in 68% of human PCas and results in activation of the PI3K pathway. We demonstrated that increased PI3K signaling via shRNA-mediated PTEN knockdown or overexpression of an activated form of AKT in murine prostate cells causes PIN lesions in the tissue-regeneration model. In this study, we combined overexpression of ERG and activated AKT and found that grafts derived from co-infected adult prostate cells weighed 2–3 times more than grafts generated from AKT or ERG overexpression alone. In contrast to AKT-induced PIN lesions, the prostate glands that simultaneously overexpressed ERG and AKT/GFP exhibited a cribriform growth pattern with cell crowding and embedded acini. The cells in these proliferative foci exhibited nuclear atypia, evidenced by hyperchromatic nuclei, mitotic figures, nuclear contour irregularity, and enlargement. These findings suggest that high levels of ERG protein collaborate with constitutively activated AKT kinase, leading to the development of invasive PCa.*
6. *High Levels of ERG Fully Transform Primary Prostate Cells Through Synergy with Enhanced AR Signaling. AR is commonly mutated or amplified in human PCa, and the AR pathway is the most extensively studied pathway in PCa because of its role in late-stage hormone-refractory PCa. Given that up-regulation of ETS transcription factors is mainly driven by the androgenresponsive TMPRSS2 promoter in most samples of human PCa, it is reasonable to hypothesize that both ETS overexpression and AR signaling coexist in malignant prostate epithelial cells.*

As we have discussed before, the subsequent work by Goldstein et al took this a step further and in murine models demonstrated the development of PIN and then PCa. However, the murine model is not exactly projectable to the human. In addition, there is no viable reverse path from HGPIN to benign cells. In fact the work of Demichelis et al indicate that watchful waiting, the proverbial do nothing strategy, is somewhat effective except in TMPRSS2:ERG fusion cases. However, the determination of the gene fusions is currently not common in prostate biopsies.

6.1.4.8 *TMPRSS2:ERG Pathways and Control*

There currently is limited pathway modeling of this fusion effect. We demonstrated the Weinberg ETS model and there is work by Yu et al showing AR control effects but no clear definitive pathway models seems to exist. A similar analysis of the AR driving of the ERG promoters is performed by Dobi et al (2010). Dobi et al conclude:

Expression of the ERG proto-oncogene, is activated in 50-70% of prostate tumors by androgen receptor (AR) mediated signals due to the fusion of AR regulated promoters (primarily TMPRSS2 and to a lesser extent SLC45A3 and NDRG1) to the ERG protein coding sequence.

Our previous studies of quantitative expression levels of ERG or TMPRSS2-ERG fusion transcripts have noted that relatively low or no ERG expression in prostate tumors significantly associated with progressive disease. Here, we have tested the hypothesis that ERG expression levels in prostate tumor cells reflect AR transcriptional regulatory function in a given biological context of the tumor progression.

Therefore, tumors with lower ERG may represent a subset with attenuated AR signaling. Expression of ERG and other AR regulated genes were evaluated Overall, ERG expression pattern was similar to that of other AR regulated genes. Strikingly low frequency of ERG expression was noted in PD tumor cells (30%) in comparison to WD tumor cells (80%), suggesting for subdued AR function in a significant fraction of tumors with genomic alterations of ERG. By integrating ERG into a panel of defined AR target genes, we developed a cumulative AR Function Index (ARFI), which if validated may have future potential in stratifying patients for targeted therapy on the basis of overall AR functional status in primary tumors....

Taken together, the ARFI approach reported here, if developed further has potential to stratify prostate tumors on the basis of in vivo functional status of AR which could lead to development of new paradigms in the treatment selection of patients for androgen ablation or other therapies. For example patients with ARFI positive versus ARFI negative/attenuated tumors may be identified in early stages of disease and latter may be more responsive to non-androgen ablation focused strategies.

Along similar lines patients with ERG gene fusion but not expressing ERG may not benefit from a potential ERG targeted therapy. Alternatively patients with varying degree of ARFI positivity may need different androgen ablation therapy strategies. Finally, association of low or no ERG in a large percentage of poorly differentiated tumors appears to be either reflection of attenuated AR signaling in tumors harboring ERG fusions or a distinct class of tumors without ERG alterations.

Clearly the ERG fusion plays a significant role in PCa. The AR effects are critical and the overall ETS pathway architecture is also a controlling element. However there is no clear and well defined path and the mechanism for the fusion seems also to be now understood at this time.

6.8.17 NKX 3.1

NKX3.1 is a gene and Nkx3.1 its protein which is putatively a tumor suppressor gene which is primarily prostate specific. As c-Myc tends to regulate the transcription of many genes, Nkx3.1 regulates the control mechanism for the prostate cells. Even more specifically it has been argued that Nkx3.1 regulates the luminal cell growth by Iwata et al. Yet Goldstein et al would argue from similar murine models that use of up-regulated ERG translocations with Akt activation,

namely putatively suppressing PTEN, allows for basal cell growth rather than luminal cell transformation.

It has been observed that diminished expression of NKX3.1 (8p21) is associated with prostate cancer progression in humans, and in mice, loss of nkx3.1 leads to epithelial cell proliferation and altered gene expression patterns....Loss of heterozygosity of 8p21 is observed in a high percentage of intraepithelial prostatic neoplasia and early carcinoma lesions, strongly implicating this region in the initial stages of prostate carcinogenesis. The importance of NKX3.1 as a dose-dependent regulator of prostate epithelial cell growth is strongly supported by analyses of nkx3.1 knockout mice . Homozygous nkx3.1 mutant mice develop prostate epithelial hyperplasia and dysplasia that progresses with age , and lesions with histologic features strongly resembling human prostatic intraepithelial neoplasia develop in homozygous mice between 1 and 2 years of age . Importantly, both hyperplasia and prostatic intraepithelial neoplasia-like lesions also occur in a significant proportion of nkx3.1 heterozygous mutants

The question then is, is NKX3.1 a true tumor suppressor gene? Despite that loss of function of Nkx3.1 predisposes to prostate cancer, it is not sufficient for tumorigenesis.(Shen 2003) Moreover, while one allele of NKX3.1 is lost by means of chromosomal deletion in PIN and prostate cancer, the other allele does not undergo mutational inactivation, although protein expression is epigenetically down-regulated or lost. These features, along with the relatively subtle consequences following forced expression of Nkx3.1 in prostate cancer cells are not consistent with activities of “classic” tumor suppressor genes, such as p53, Rb, or Pten. Instead, Nkx3.1 appears to act more like a tumor modulator, serving as a regulator of differentiation, which in turn prevents cancer initiation. In this regard, further analyses of NKX3.1 can provide important insights into the relationship between regulation of differentiation and carcinogenesis.

As Iwata et al have observed:

The prevailing model of NKX3.1 expression in human prostate cancer suggests that while the protein may decrease in PIN lesions, it is much more commonly decreased in invasive adenocarcinomas, and nearly completely lost in most, if not all, metastatic prostate adenocarcinomas ... there was a variable decrease in expression of Nkx3.1 in PIN lesions, and that Nkx3.1 was virtually completely lost in invasive adenocarcinomas... several observations from our group regarding NKX3.1 differ from this prevailing view. First, in a previous report, while reductions of NKX3.1 protein occurred in PIN lesions and some adenocarcinomas, the reductions were relatively minor and virtually all invasive adenocarcinomas retained significant levels of NKX3.1 protein ...

More recently we have found that the majority of very high grade (Gleason score 8–10) localized prostate cancers ... retain high levels of expression of NKX3.1 protein. In the present study we found that, as compared to high grade PIN, the staining for Nkx3.1 protein actually increased substantially in pre-invasive cribriform PIN/CIS lesions and in early invasive adenocarcinomas, and these levels correlated inversely with levels of MYC expression.

These results indicate the Nkx3.1 may be dynamically regulated during progression of this disease. ... It is possible, therefore, that Nkx3.1 expression in invasive prostatic acini in MYC-

driven mouse prostate cancers may represent a recapitulation or caricature of the process of stromal invasion/branching morphogenesis in development, and, that Nkx3.1 may facilitate this process. ...

Lei et al. found that forced restoration of Nkx3.1 expression in Pten null epithelium led to decreased cell proliferation, increased cell death, and prevention of tumor initiation .. They further showed that Nkx3.1 was required to engage the p53 pathway, indicating that reduced Nkx3.1 expression can itself abrogate p53 signaling.

These findings raise the interesting possibility that the reduction in Nkx3.1 seen upon the induction of MYC in the mouse prostate prevents the induction of p53 induced apoptosis, thus facilitating MYC's ability to transform these cells. Additional studies in which Nkx3.1 expression is kept at high levels during induction of MYC in prostate epithelium will be required to address this question further. We do not know precisely how MYC is regulating Nkx 3.1 protein expression, ... ”

Specifically Iwata et al state:

Since MYC may downregulate Nkx3.1 at the level of transcription ..., it is possible that elevated MYC itself may be responsible for down-regulating Nkx3.1 expression.

In effect, this implies that MYC controls NKX3.1 and thus up-regulated MYC results in a down regulated NKX3.1. If NKX3.1 is controlling prostate stability then its overall regulation is via MYC. Controlling and suppressing MYC would control and up-regulate NKX3.1 and thus stabilize prostate growth. The complete pathway for this gene does not seem to be complete at this stage. Its importance is well defined however.

6.8.18 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.

6.1.4.9 *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: **Platelet and LEukocyte C Kinase** 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 $\beta\gamma$ -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.

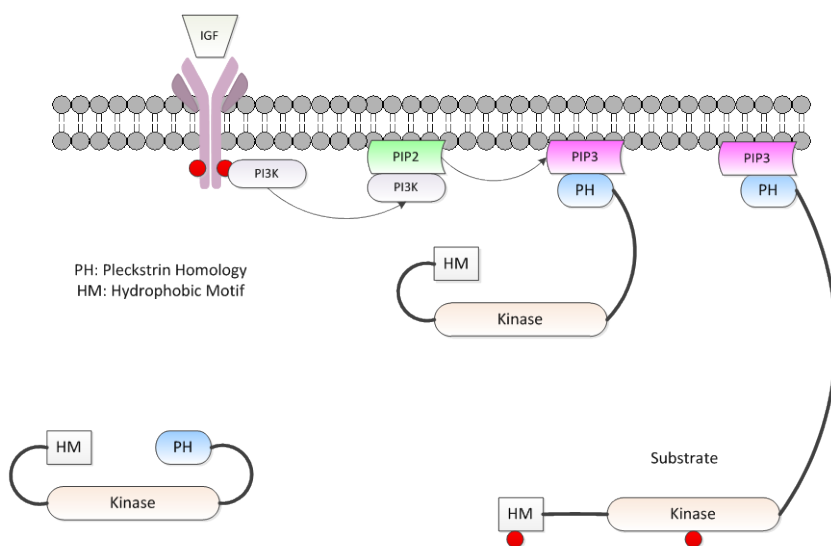
PH domains can be found in many different proteins, such as ARF. Recruitment to the Golgi in this case is dependent on both PtdIns and ARF. A large number of PH domains have poor affinity for phosphoinositides and are hypothesized to function as protein binding domains. Proteins reported to contain PH 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 NrK 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, dbp, 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.

6.1.4.10 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.



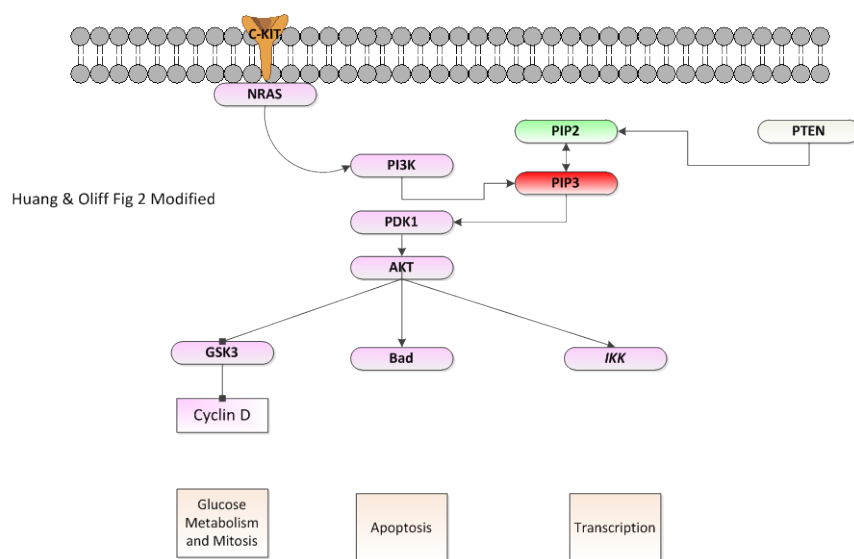
From: Marks et al, p 178.

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 tuberlin (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(3,4,5)P₃ 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 phosphoinositide-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)P₃ and PtdIns(3,4)P₂, 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.

6.1.4.11 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 IC₅₀ values of 2.7 and 21 μ M respectively. Neither compound inhibited Akt3 nor mutants lacking the PH (pleckstrin homology) domain at concentrations up to 250 μ M.

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 Thr₃₀₈ and Ser₄₇₃, 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 be 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.

6.8.19 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 Q61L-transformed 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^{3,4} 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.

6.9 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- κ B
- TCF
- TCF
- TOR

6.9.1 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.

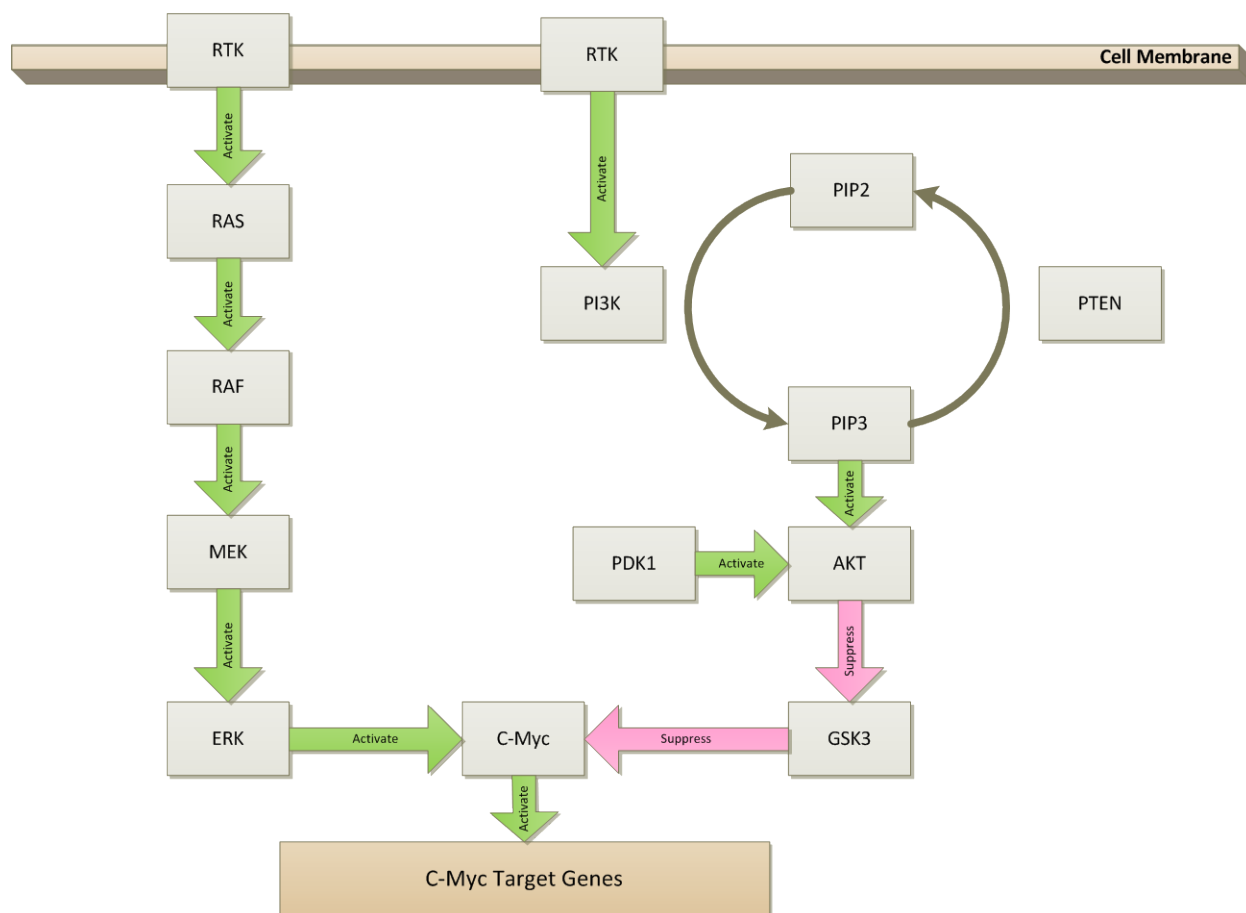
Our results using IHC provide novel insights that support the contention that MYC overexpression is sufficient to transform prostate luminal epithelial cells into PIN cells in vivo. We also identified a novel histopathologically identifiable intermediate step prior to invasion that should facilitate 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. In 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 influenced 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.

6.9.2 CDK

6.9.3 MITF

MITF (microphthalmia 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 allows 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, 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 naevus count and non-blue eye colour. Functional analysis of E318K showed that MITF encoded by the variant allele had impaired sumoylation 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.

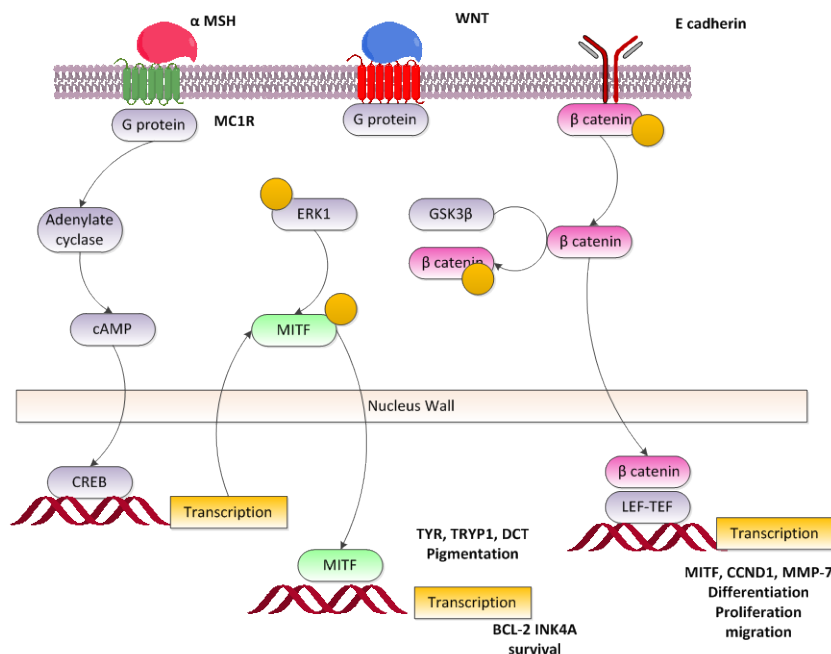
Decreased or absent pigmentation and decreased or absent expression of SILV and MLANA accompany the progression from nevus to melanoma.

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 GSK3 β , 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 p21^{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.

6.9.4 NF-κB

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.

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.

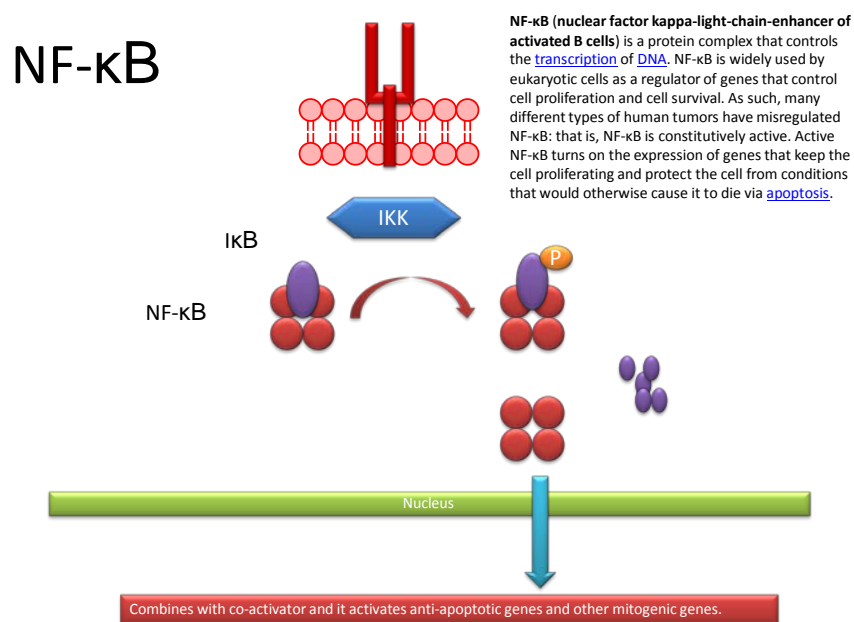
Nuclear Factor (NF)-κB is a family of transcription factors composed of homo- and hetero-dimers initially identified as an enhancer binding protein for the immunoglobulin light chain in B lymphocytes...

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- κ B/p65 expression is predictive of biochemical recurrence in patients with positive surgical margins after radical prostatectomy and nuclear localization of NF- κ B is increased in prostate cancer lymph node metastasis and can be used to predict patient outcome. These results demonstrate that NF- κ B/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- κ B is clearly an activator of an anti-apoptosis process in the nucleus. The paper by Huang et al shows that blockade of NF- κ B is an effective suppressor of angiogenesis, invasion and metastasis of prostate cancer.



2/1/2011

Prostate Genetics and Dynamics

21

NF- κ B is another transcription protein seen in melanoma. This protein is characterized by:

- NF- κ B 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- κ B appears on 10q24 and is somatic and acts in a dominant manner.

NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) is a protein complex that controls the transcription of DNA. NF- κ B 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- κ B: that is, NF- κ B is constitutively active. Active NF- κ B 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- κ B) is an inducible transcription factor that regulates the expression of many genes involved in the immune response. Recently, NF- κ B activity has been shown to be upregulated in many cancers, including melanoma. Data indicate that the enhanced activation of NF- κ B may be due to deregulations in upstream signaling pathways such as Ras/Raf, PI3K/Akt, and NIK. Multiple studies have shown that NF- κ B is involved in the regulation of apoptosis, angiogenesis, and tumor cell invasion, all of which indicate the important role of NF- κ B 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- κ B is an emerging hallmark of various types of tumors including breast, colon, pancreatic, ovarian, and melanoma [9–14]. In the healthy human, NF- κ B 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- κ B also modulates the expression of factors responsible for growth as well as apoptosis. However, increased activation of NF- κ B 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- κ B'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- κ B plays an important role as a mediator of inhibition of apoptosis. In melanoma, NF- κ B 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- κ B 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 κ B (NF- κ B), which regulates the expression of many anti-apoptotic, pro-proliferative and pro-metastatic genes.

Canonical activation of the NF- κ B pathway occurs when NF- κ B switches its localization from the cytoplasm, where it is maintained inactive by assembly with the inhibitor I κ B protein, to the nucleus, where NF- κ B regulates gene expression. NF- κ B activation relies upon the phosphorylation dependent ubiquitination and degradation of I κ B mediated by the I κ B kinase (IKK) complex and β -Trcp E3 ubiquitin ligases.

Consequently, both IKK activity and the levels of β -Trcp regulate the extent of I κ B degradation and hence NF- κ B activation. The genetic basis that underlies the elevated NF- κ B activity in malignant melanoma largely remains elusive.

Constitutively active IKK has been demonstrated to sustain NF- κ B activation in human melanoma cells, resulting in induction of the chemokine CXCL1. CXCL1, in turn, is capable of activating IKK and NF- κ B 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- κ B ..., the mechanism(s) by which BRAF_{V600E} (BRAF_{VE}) may elicit NF- κ B signaling in melanoma cells have not yet been elucidated. Activation of the canonical NF- κ B 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- κ B 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- κ B.

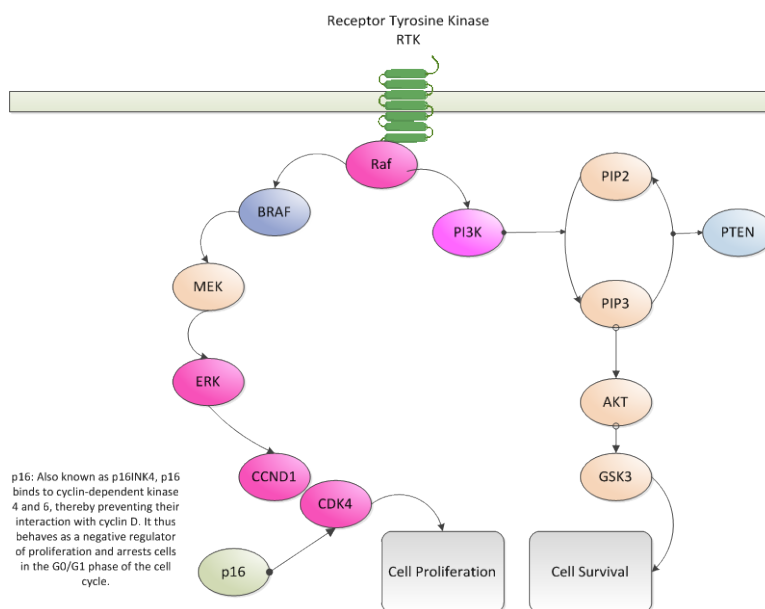
6.10 PATHWAYS AND IMMUNE RESPONSE

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.

6.10.1 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.

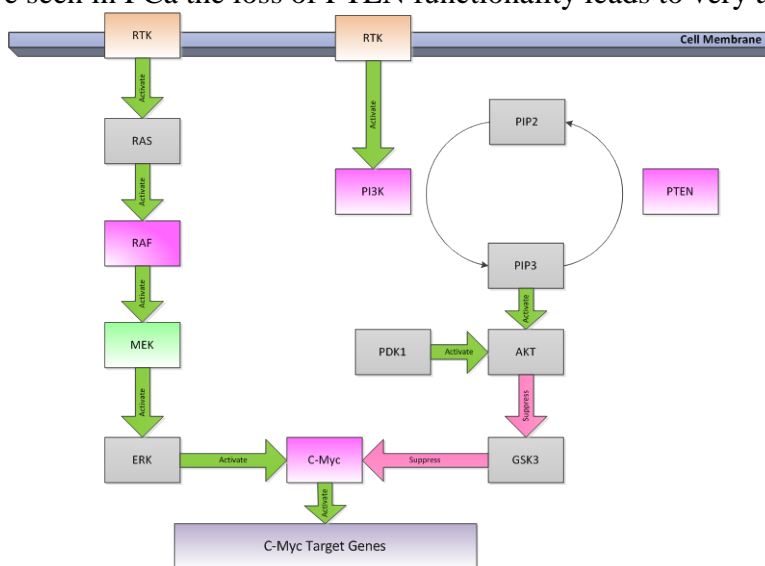
The growth factors activate the RTK kinase which activates the RAS which activates a B-RAF which overexpresses its product and this over-expression is what drives the proliferation pathway. It is this single gene and its protein expression which causes the problem in 60% of the cases.

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.

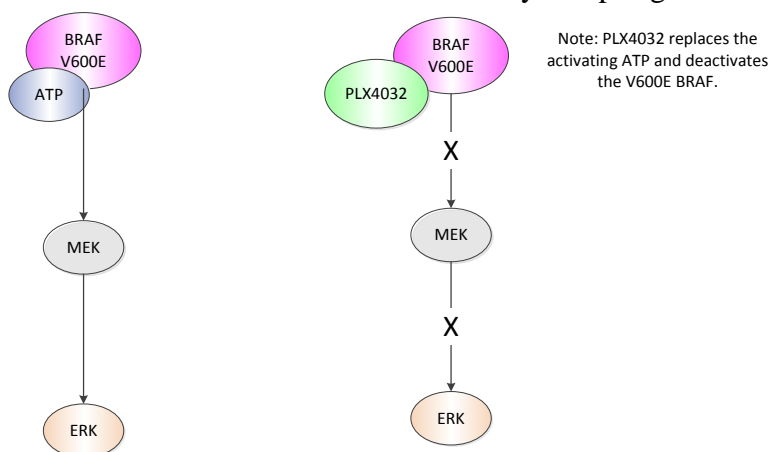
1. Using a methodology such as microarrays, attempt to identify genes, or their expressions, which are present in the malignant cell. These are not unique and sometimes they are transient as well. The B-RAF identification is an example.
2. Develop a target molecule which can attach to and inactivate 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.

6.10.2 B-RAF control

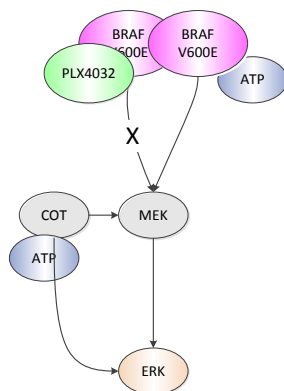
The most recent one is the control of a mutated B-RAF, a variant of the RAF pathway. It was observed that there was a mutation in the B-RAF gene so that what was produced was a different B-RAF called V600E which had excessive up-regulation in almost 50-60% of all metastatic melanomas. The identification 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 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. PDGFR β 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 ...*

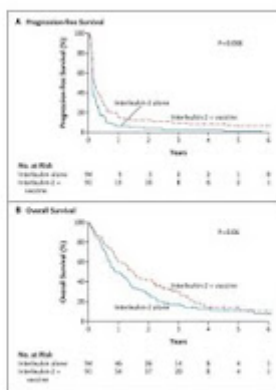
(ii) *The findings of Johannessen et al. suggest another mechanism for the resistance of ERK signaling to RAF inhibition in cells driven by the BRAF V600E mutations. These investigators used a new technique — the introduction of a library of DNA constructs, each of which encodes a different kinase — 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. ⁸They further showed that the overexpression of mitogen-activated protein 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.*

6.10.3 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.



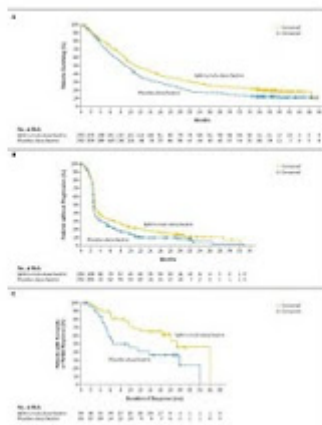
It should be noted that there is some improvement but still there is a very poor survival prognosis.

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.

6.10.4 Considerations

The current efforts clearly show some significant advancement. However there are several key issues which must be clarified:

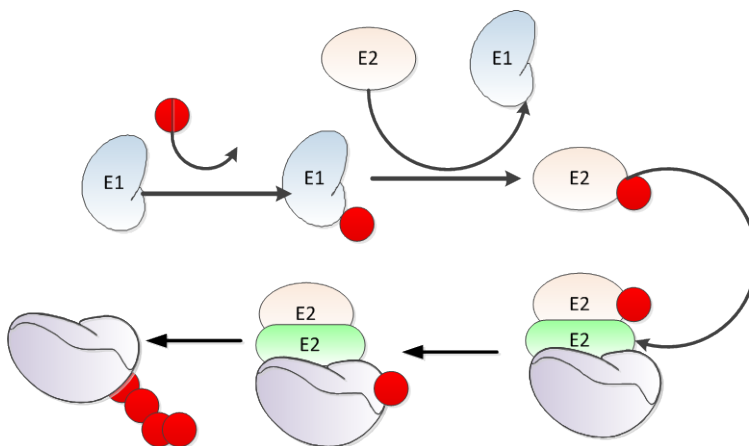
1. Is melanoma like colon cancer as described by Vogelstein or do we have a somewhat random set of mutations depending on the location of the lesion. Namely is melanoma really a disparate set of different sub-cancers. Is there a clear genetic pathway, is there a gene that predisposes and if so how. The 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.

6.11 UBIQUITINATION

The normal destruction, consumption would be better, of cell proteins is performed by ubiquitin. The process is called Ubiquitination. The process is explained graphically below.



6.12 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? Frankly 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.

6.13 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.

| <i>Element</i> | <i>Type (Ligand, Receptor, Cell Surface, Pathway, Intra Nucleus, Transcription)</i> | <i>Function http://www.ncbi.nlm.nih.gov/gene/</i> |
|----------------|---|---|
| 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 <i>Drosophila</i> , 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 has 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, tumorigenesis, 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 is 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 <i>ABL1</i> 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 G₁/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 |

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| 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 <i>FRAP1</i> 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 |

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| p21 | Pathway | <p>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.</p> |
| p27 | Pathway | <p>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.</p> |
| p53 | Pathway | <p>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</p> |

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| 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 | <i>PREX2</i> 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. |

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| 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 <i>SPOP</i> 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. |

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| 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 tuberlin. 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), pilomatixoma (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 <i>ERBB4</i> 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 phosphatidylinositol-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 . |

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| 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 leukemia, and piebaldism. |

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| 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 <i>Drosophila</i> , 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. |
| Smoothed | 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 transduces 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 <i>S. cerevisiae</i> cdc28 and <i>S. pombe</i> 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). |

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| 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 <i>ETV1</i> 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 |

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| 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 |

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| 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. |

6.14 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.

| <i>Gene</i> ⁷² | <i>Location</i> | <i>Function</i> |
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| 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. |

⁷² <http://www.ncbi.nlm.nih.gov/gene/6794> we use this source for gene descriptions.

| <i>Gene</i> ¹² | <i>Location</i> | <i>Function</i> |
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| 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 tuberlin. 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. |

| <i>Gene</i> ¹² | <i>Location</i> | <i>Function</i> |
|---------------------------|-----------------|--|
| 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 is 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. |

| <i>Gene</i> ¹² | <i>Location</i> | <i>Function</i> |
|---------------------------|-----------------|---|
| 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 |

| <i>Gene</i> ¹² | <i>Location</i> | <i>Function</i> |
|---------------------------|-----------------|--|
| 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), pilomatixoma (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. |

| <i>Gene</i> ¹² | <i>Location</i> | <i>Function</i> |
|---------------------------|-----------------|--|
| 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 has 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. |

7 PROSTATE STEM CELLS

There has been a great deal of work on stem cells. We may think of such cells as being part of the embryo, and in the placenta at birth. They are thought of as the universal cell generator. Theoretically the stem cell should become whatever cell type we may want it to be. In a more narrow sense there may be a variety of localized stem cells, namely cells which replenish local cells which are worn away such as on the skin or in the colon. It is not the mature cells which do the reproducing but it is the few stem cells which reside in say the basal layer of the skin which reproduce and create off spring which are just plain old keratinocytes.

In this Chapter we examine in some detail the prostate stem cell, and in turn we generate the ability to consider the cancer stem cell issue in broader detail.

The focus is on stem cells. It does not address the pathways which are different or activated. That in itself is a critical question. Namely what differentiates a stem cell from a mature non stem like cell when we examine the pathways? Thus when looking at PCa we see that pathway changes are then most likely pathway changes in the stem cell alone, yet if the agglomeration of stem cells is such that the non-stem constituents reflect the genetic makeup of the stem cell, then we would expect some parity in pathway dynamics. This will be an issue we examine in a later report.

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 it 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 manner⁷³. 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, research⁷⁴:

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' 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. In the above definition, there is the issue of what makes the stem cell different and how many are there and how do we identify it. The CSC is in one sense the single cell which can regenerate a full cancer growth. But does that mean in vivo or in vitro or both? Murine models have been used extensively but there are serious questions regarding their extensibility.

We shall discuss some of these issues in this report. 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."

⁷³ Weinberg, Cancer, pp 416-417.

⁷⁴ <http://www.cancer.gov/ncicancerbulletin/072710/page4>

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.

The above has substantial positive and negative impact. A single stem cell may control everything, for a while. If however it undergoes mitosis then we may have many stem cells. Or we may keep a single one. For example if a stem cell in mitosis reproduces a single stem cell plus a non-stem cancer cell, then we maintain single CSCs, while we multiply the malignant non CSC cells. However, if the CSC in mitosis just multiplies itself for a while, then we end up with a collection of very powerful and spreadable bombs of CSCs.

The NCI also continues:

“The reason why it’s so much stronger for hematologic malignancies are 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.”

The existence of CSCs in PCa 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.

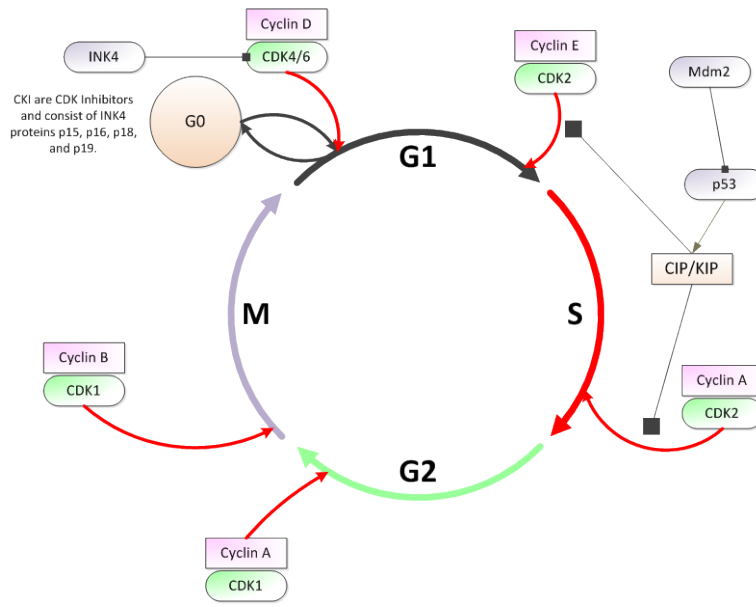
Many other such issues will arise and we discuss the CSC idea here and we return to it later in the work.

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 PARADIGM

The first issue is a definition of a stem cell. We may understand stem cell from the hematopoietic stem cells found in the bone which give rise to a variety of blood cells and other types of cells. In fact almost all cells in the body which require some form of replenishment have such stem cells. Consider the skin. The basal layer has stem cells to generate the keratinocytes. In fact it may be argued that melanocytes have their own stem cells as well.

Cells are reproducing via the cell cycle as we show below and discuss in Appendix B. With a stem cell, it is only that cell which does the mitotic division; all other cells are just mature functioning cells subject to normal cell death or apoptosis.



The question is however, which cells. Which cells are the stem cells? Are all cells reproducing or just some select class of cells. The concept of stem cells makes the issue one of a small select group of cells. These are the stem cells.

As Alberts et al state (pp 1417-1421):

Humans renew the outer layers of their epidermis a thousand times over in the course of a lifetime. In the basal layer, there have to be cells that can remain undifferentiated and carry on dividing for this whole period, continually throwing off descendants that commit to differentiation, leave the basal layer, and are eventually discarded.

*The process can be maintained only if the basal cell population is self-renewing. It must therefore contain some cells that generate a mixture of progeny, including daughters that remain undifferentiated like their parent, as well as daughters that differentiate. Cells with this property are called **stem cells***

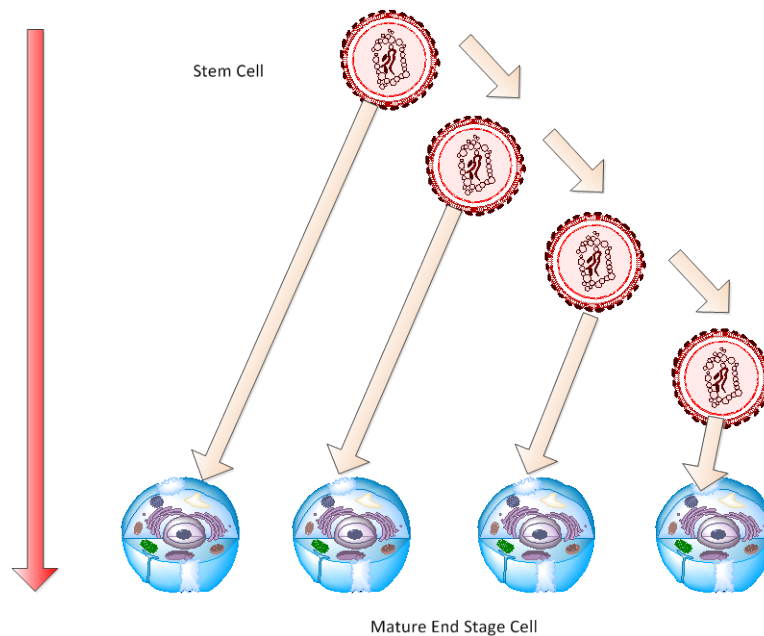
They have so important a role in such a variety of tissues that it is useful to have a formal definition. The defining properties of a stem cell are as follows:

- 1. It is not itself terminally differentiated (that is, it is not at the end of a pathway of differentiation).*
- 2. It can divide without limit (or at least for the lifetime of the animal).*
- 3. When it divides, each daughter has a choice: it can either remain a stem cell, or it can embark on a course that commits it to terminal differentiation.*

Stem cells are required wherever there is a recurring need to replace differentiated cells that cannot themselves divide. The stem cell itself has to be able to divide—that is part of the

definition—but it should be noted that it does not necessarily have to divide rapidly; in fact, stem cells usually divide at a relatively slow rate.

We present below a simplified example of a specialized stem cell. The stem cell is the only one of its kind to divide. The mature cells do not generally divide; they are just functional and proceed to mature. The stem cell always produces at least one of its own kinds, another stem cell, and then one of the mature like cells. Note the initial stem cell. In this example we allow it to divide and produce one stem cell and one maturing cell. Thus at some point this process just keeps the number of stem cells constant but can produce an ever growing number of maturing cells.



Now when we examine the above we can see that if the stem cell divides once every hour, and the life of a mature cell is say 24 hours, then we have a growth effect. We must have a cell stability of one replenishment per one destroyed. During a growth state however, the stem cells are reproducing quickly and cells are added. The stem cell responds to surface stimulants to enter into cell cycle production.

As Tang et al state:

Normal adult stem cells (SC) have several fundamental properties: they are generally very rare, can self-renew, have tremendous proliferative potential but normally (i.e., in their niches) are quiescent, and can differentiate along one or several different cell lineages.

The most defining property of a SC is its ability to self-renew while being able to differentiate into all different lineages of progeny and even to reconstitute an organ, as exemplified by a single hematopoietic SC (HSC) to reconstitute the whole blood and rescue an irradiated mouse. SC development is a continuous and dynamic process, in which cells with distinct self-renewal, proliferative, and differentiation abilities may co-exist.

For example, mouse HSC are heterogeneous populations of cells containing long-term HSC (LT-HSC), which can sustain life-long self-renewal and reconstitution, and short-term HSC (ST-HSC), which can sustain self-renewal and reconstitution for only 8 wk. The ST-HSC generate multi-potent progenitor (MPP) cells exhibiting only limited self-renewal capacity, which then further develop into lineage-restricted progenitor (or precursor) cells that have lost self-renewal ability.

Although this paradigm of LT-HSC/ST-HSC early progenitors (MPP) late progenitors differentiated cells in mouse bone marrow can, in principle, be applied to other SC developmental processes, in reality, little is known about most tissue SC lineages and we often name the subsets of cells in a specific tissue/organ with certain self-renewal and differentiation abilities simply stem/progenitor cells. Such is the case with the putative prostate epithelial stem and progenitor cells.

Consequently, throughout this review, we shall frequently use the term ‘(prostate) stem/progenitor cells.’

The above feature of maturing into various lineages is clearly seen in blood cells but one may question just where it functions say in prostate cells. Is there a single stem cell which generates either a basal or luminal cell or if so where does it reside, and how does this differentiation occur? This is the point made by Tang et al towards the end of the above quote.

Cancer stem cells are a variant of the benign stem cell. Namely a cancer stem cell is a cell which behaves like a stem cell in terms of cell proliferation but now has genetic changes which reflect malignant behavior. In an NIH report the authors 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.

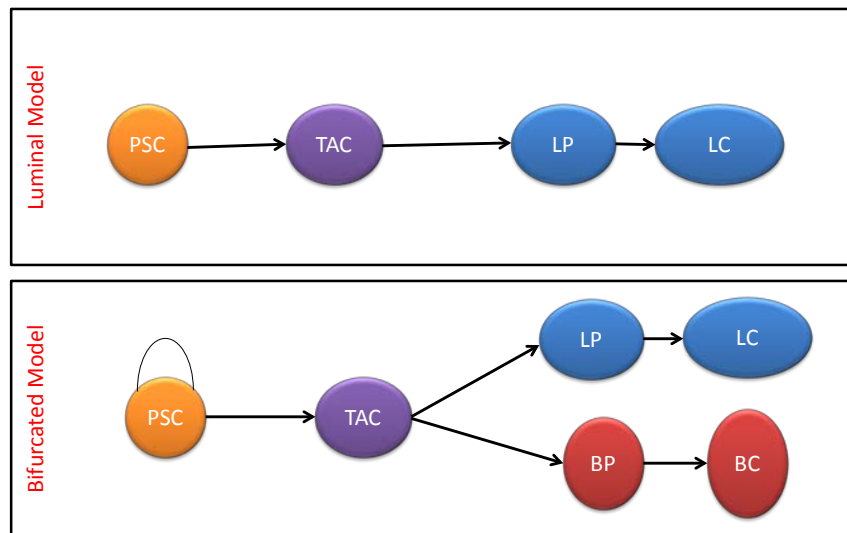
The CSC hypothesis therefore does not imply that cancer is always caused by stem cells or that the potential application of stem cells to treat conditions such as heart disease or diabetes, as discussed in other chapters of this report, will result in tumor formation. Rather, tumor-initiating cells possess 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.

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.



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 non-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.

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 are 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 dynamics⁷⁵. 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.

Nature has an interesting poster on the cancer stem cell, CSC⁷⁶. The poster states:

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

⁷⁵ http://www.eurekalert.org/pub_releases/2011-01/uof-gfm012011.php

⁷⁶ http://www.nature.com/nrc/posters/cancerstemcells/csc_poster.pdf

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 theory⁷⁷:

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 genes (Ephb2), which encodes a receptor tyrosine kinase, the leucine-rich repeat-containing G protein-coupled

⁷⁷ <http://stm.sciencemag.org/content/3/81/81ec64.short?rss=1>

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.

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.

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 arise Transit Amplifying Cells and then the PMDC.

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.

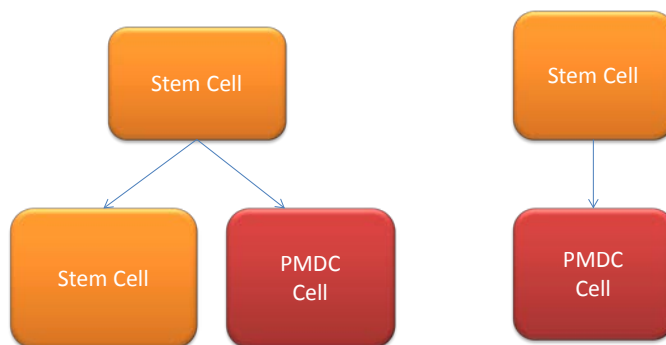
Finally when we return to the HGPIN model we see the benign cell migrating to a dysplasia, say HGPIN, and then to a malignant cell, but then there is the regression back to a benign cell. The question is then; what pathway elements take us one way and what elements take us back. And what happened to 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.

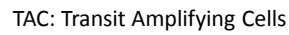
7.2 PROGRESSION AND REGRESSION

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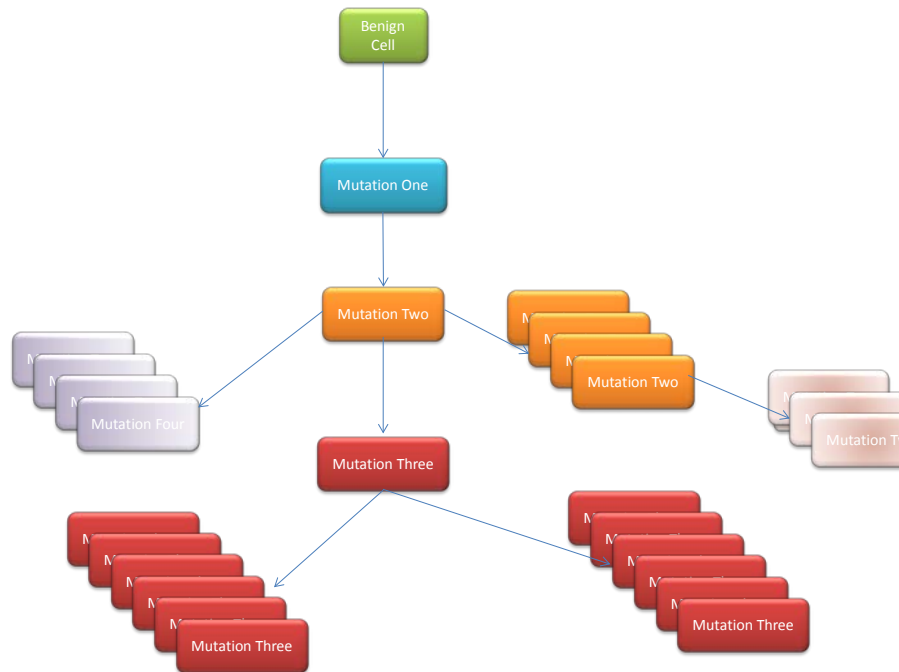
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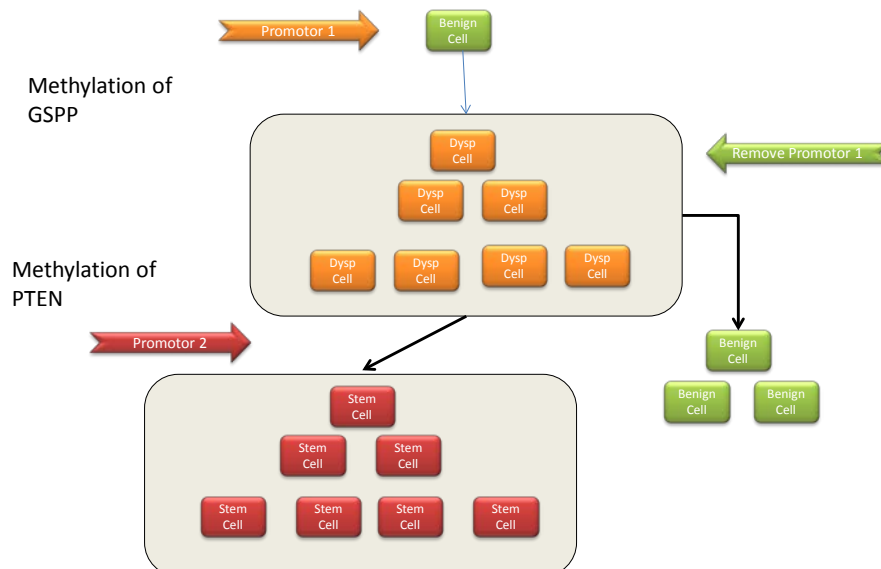
PMDC Cell: Post Mitotic Differentiated Cancer Cell



307 | Page



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7.3 STEM CELL COMPLEXITY

Recall as we have indicated 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, says 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. First 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 intra 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.

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 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 a 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 have 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.

7.4 PCA STEM CELL RECOGNITION

Recent work by Qin et al. examines the more detailed nature of the prostate cancer stem cell (PCa CSC). We here look at that as a starting point and then examine some of the surrounding literature to see if the results from that work can be extensible. The cancer stem cell model is one which akin to the stem cell model above states that there are classes of stem like cells which have been mutated and the development of cancer results from the turning on of these cells.

Before proceeding let us review a few issues. It should be noted that we are simplifying the analysis to intensify several points and let the reader focus on the literature to assist in resolving some of the lost complexities. Now:

1. Stem cells have certain characteristic and the only one we focus on here is that for the most part they are the only cells of a class which have the ability to reproduce. In a stable environment, the stem cells reproduce at a rate equal to the loss of mature functional cells. Thus in the skin, the basal stem cells reproduce at a rate equivalent to the death and loss of the keratinocytes, no more or less. Let there be an injury then they produce more by being activated by some ligand on some receptor on the stem cell. Cells reproduce until equilibrium is reached.
2. Mature cells, derivative from stem cells, do not reproduce. They just do what they were intended to do, no more or less.

As Wang and Shen state in a recent article (2011):

The cancer stem cell (CSC) model proposes that cells within a tumor are organized in a hierarchical lineage relationship and display different tumorigenic potential, suggesting that effective therapeutics should target rare CSCs that sustain tumor malignancy...CSCs are instead defined in practical terms through the use of several functional assays. The most frequently used methodology involves xenotransplantation of flow sorted populations of primary cancer cells into immunodeficient mice. In this assay, CSCs are defined as a subpopulation of cells within a primary tumor that can initiate tumor formation in mice following transplantation, unlike the remaining tumor cells

This is a definition limited to the assay produced. It is not a broad based definition.

Wang and Shen then discuss the types of prostate cells:

In human and mouse, the normal prostate gland epithelium contains three primary differentiated cell types.

1. *Luminal cells are columnar epithelial cells that express secretory proteins as well as markers such as cytokeratin 8 (CK8), CK18, Nkx3.1, prostate-specific antigen and high levels of androgen receptor (AR).*
2. *Basal cells are localized beneath the luminal layer and express markers including CK5, CK14 and p63, but express low levels of AR.*

3. *A rare third type of cells termed neuroendocrine cells express endocrine markers such as synaptophysin and chromogranin A, but do not express AR.*

Then they allege:

Prostatic intraepithelial neoplasia (PIN) is often considered a precursor of prostate cancer, and is characterized histologically by luminal epithelial hyperplasia and a progressive loss of basal cells ...

Here we have previously expressed concern regarding counter-examples. Namely it is known that there are patients where a diffuse HGPIN may be present upon a high density sampling and then after a second high grade sampling the HGPIN is totally gone. The question is why? If as many agree HGPIN is the precursor of PCa and if moreover HGPIN is already a representation of a CSC mutation, then what has reversed the mutation. Perhaps it was the fortuitous removal on the CSC in the initial sampling? We have argued that such may be inductively deduced from examining the number of times this occurred related to the statistical chance of such happening.

In a recent paper, Qin et al state⁷⁸:

Prostate cancer (PCa) is heterogeneous and contains both differentiated and undifferentiated tumor cells, but the relative functional contribution of these two cell populations remains unclear. Here we report distinct molecular, cellular, and tumor-propagating properties of PCa cells that express high (PSA⁺) and low (PSA^{-/lo}) levels of the differentiation marker PSA. PSA^{-/lo} PCa cells are quiescent and refractory to stresses including androgen deprivation, exhibit high clonogenic potential, and possess long-term tumor-propagating capacity.

They preferentially express stem cell genes and can undergo asymmetric cell division to generate PSA⁺ cells.

Importantly, PSA^{-/lo} PCa cells can initiate robust tumor development and resist androgen ablation in castrated hosts, and they harbor highly tumorigenic castration-resistant PCa cells that can be prospectively enriched using ALDH⁺CD44⁺α2β1⁺ phenotype.

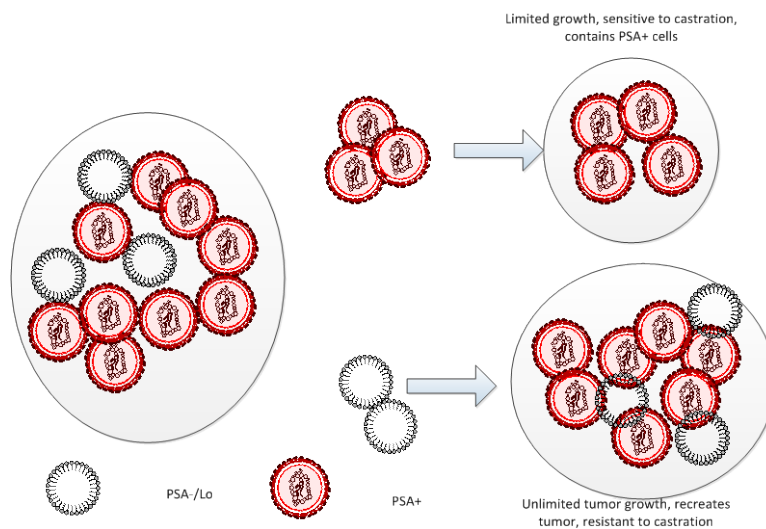
In contrast, PSA⁺ PCa cells possess more limited tumor-propagating capacity, undergo symmetric division, and are sensitive to castration. Altogether, our study suggests that PSA^{-/lo} cells may represent a critical source of castration-resistant PCa cells.

Specifically:

1. *PSA^{-/lo} PCa cells are quiescent and refractory to anti-androgen and chemotherapy*
2. *These cells express stem cell genes and can undergo asymmetric cell division*
3. *They also possess long-term tumor-propagating capacity in intact male mice*
4. *PSA^{-/lo} PCa cells are highly tumorigenic and resist androgen ablation in vivo*

⁷⁸ <http://www.cell.com/cell-stem-cell/abstract/S1934-5909%2812%2900126-9>

We depict the details from the paper and show it below:



As Merville states in commenting on the work of Qin et al⁷⁹:

In cell lines and mouse model experiments, the low-PSA cells resisted chemotherapy and thrived under hormone deprivation, the two main prostate cancer drug treatments, the researchers found.

Low-PSA cells were found to be both self-renewing and capable of differentiating into other prostate cancer cell types upon division, a hallmark of stem cells called asymmetric cell division. "Asymmetric cell division is the gold standard feature of normal stem cells," Tang said. "Using time-lapse fluorescent microscopy, we were able to show asymmetric cell division by filming a low-PSA cell dividing into one high-PSA cell and one low-PSA cell."

When the team implanted the two cell types in hormonally intact male mice, the rapidly reproducing PSA-positive cells caused faster growth and larger tumors in the first generation. However, after that the low-PSA cells generated larger, faster-growing tumors and tumor incidence in the high-PSA cells dropped.

In fact, the low-PSA prostate cancer cells possess indefinite tumor-propagating capacity. In contrast, when implanted in the castrated mice, the low-PSA prostate cancer cells developed much larger tumors than the corresponding high-PSA cells. In another experiment, mice with tumors generated by either cell type were then castrated and treated with hormonal therapy.

Low-PSA tumors grew better in these doubly androgen-deprived mice than the high-PSA tumors. "These findings closely resemble progression observed in patients after androgen-deprivation treatment and reflect reduced PSA-producing cells in patient tumors after androgen depletion," Tang said.

⁷⁹ http://www.eurekalert.org/pub_releases/2012-05/uotm-sip050412.php

As Jeet et al state regarding their view of the prostate related stem cell:

Stem cells are unspecialized cells that can self-renew and differentiate to yield a diverse range of specialized cell types of a tissue or organ. The mouse prostate comprises dorsal, lateral, ventral, and anterior lobes, each containing three regions of proliferating cells—distal, intermediate, and proximal. It has been suggested that the prostatic stem cells reside in the proximal region of the mouse prostate.

These findings, together with tissue recombination approaches (that allow the study of mesenchymal-epithelial interactions in developing tissues), led to the elegant work that developed a new prostate regeneration system by combining CD117 (a prostate stem cell marker predominantly expressed in the proximal region) positive fractions from C57BL/6 mouse donors with rat embryonic urogenital sinus mesenchymal stromal cells. These cells were then placed under the renal capsule of athymic nu/nu mouse hosts to generate functional, secretion-producing prostates. This is the first model to demonstrate the ability of mesenchyme to trigger prostate genesis thus opening up possibilities for developing insights into the earliest changes that evolve into cancer.

Jeet et al argue that their work demonstrates the ability of these identified stem cells to have a form of prostate related pluripotency. They like many others have been using cell markers as a means of tracking the stem cell. One may then ask what is the cell receptors and activating ligands which result in the stem cell ability to perform its regenerative functions.

As Zhang stated:

Importantly, Staeger and Max also noted that tumor stem cells in EFT have been identified. These tumor stem cells expressed some markers of embryonic stem cells. There are cell populations with the phenotype of embryonic stem cells in the adult body. It remains unclear as to whether such cell populations are permissive for EWSR1-FLI1 induced transformation and whether EFT is derived from these cell populations.

Zhang has extended this identification somewhat but the issue of good markers remains.

Yet as Gupta et al state:

Some of the controversy surrounding the CSC model seems to arise from confusion regarding the definition of CSCs, leading to two key objections against the use of this term.

The first objection derives from the fact that, unlike the case for normal stem cells, which are usually oligo or multipotent, it is currently unclear whether CSCs can give rise to multiple differentiated cell types....

A second key objection to the CSC model is that it is currently unclear whether the normal cellular precursors of CSCs are, in fact, bona fide stem cells. It is clear, however, that the traits used to define CSCs do not rely on knowledge of their cellular origins within normal tissues. Accordingly, the CSC model must stand or fall on the basis of experimental characterizations of cancer cell populations

The Gupta et al observations are quite important. Namely, is a stem cell born or made. Namely is there an unbroken lineage from stem cell to stem cell? Also his first observation is the pluripotency issue, namely, is stem cells able to generate a broad number of cells or is stem cells cell-specific? The current nature of the Gupta et al observations does raise issues as to how well we understand the stem cell model.

As Tang et al conclude:

The hypothetical model of hierarchical organization of PCa cells has several important implications. Above all, it can help explain how the tremendous heterogeneity associated with the PCa can be generated. The rare PCa SC that persist in a tumor will continue to generate a repertoire of progenitor cells that in turn will develop into a spectrum of cells at different stages of differentiation, thus engendering the heterogeneous phenotype of the tumor. The model posits that the tumorigenic stem/progenitor cells are mostly undifferentiated cells as supported by the observations that most CD44 and CD133 cells are AR. The model also implies that most differentiated, luminal-like cells, which constitute the bulk of the tumor, might be much less or even non-tumorigenic (Figure 6A). In support, prospectively purified CD57 cells are non-clonogenic and non-invasive [44] and prospectively purified PSA_b cells are less tumorigenic than the isogenic PSA_a cells.

They also note the positive and negative PSA in the prior paper by Tang.

There is a great deal of concern as regards to where the stem cells come from. Namely the issue of the cells of origin. Previously we had reviewed the Goldstein model, where they had indicate a basal stem source as compared to a luminal cell source.

Wang and Shen state:

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 hierarchy. 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.

They consider several sources. For basal cells they state:

Although prostate tumors display a strongly luminal phenotype, this does not exclude the possibility that basal cells could be a cell of origin for prostate cancer. In particular, it is

possible that transformed basal cells could differentiate to generate large numbers of luminal cancer cells. For example, prostate-specific conditional deletion of Pten by a probasin-Cre driver allele has been shown to result in a basal cell expansion accompanied by increased number of intermediate cells, suggesting a basal cell of origin ... An important recent study from the Witte laboratory has used similar approaches with primary human prostate tissues to show that basal cells are a cell of origin for human prostate cancer

The Witte lab results are those of Goldstein et al which we have discussed at length (See Appendix A).

In contrast we have luminal cell origin as stated as follows:

Other studies have provided evidence that luminal cells can serve as cells of origin for prostate cancer. For example, pathological analysis of high-grade PIN samples, which still retain basal cells, suggest that molecular events associated with human prostate cancer initiation such as upregulation of c-MYC and shortening of telomere length occur exclusively in luminal cells but not their basal neighbors ...

In Moscatelli and Wilson, the authors state:

There is nothing inherently contradictory in the results described by Wang et al. and Goldstein et al., because it is possible that both basal and luminal stem/progenitor cells may independently serve as cells of origin for prostate cancer.

Indeed, it is also possible that oncogenic stimuli may differ in their effectiveness in transforming distinct cell populations. The tumors that arise from different target cells may also vary in their biological behavior and genetic profiles.

There are also indications that normal prostate stem cells may reside in both the basal and the luminal compartments. Thus, if stem cells are preferentially targeted during malignant transformation, both compartments may contain cells of origin for prostate cancer.

Most of the scientific evidence indicates that prostate stem cells reside in the basal layer and give rise to the secretory luminal cells via transit-amplifying cells, which are intermediate in phenotype between stem cells and terminally differentiated cells.

There is definitive evidence that

(i) secretory cells of the adult murine prostate derive from cells that express p63, a transcription factor that is expressed by all basal cells in the prostate , and

(ii) p63- expressing basal cells are required for prostate development. In addition, prostate basal cells (human and murine) have greater proliferative activity in vitro and in vivo than luminal cells.

The molecular signature of prostate stem cells also identifies a basal-like phenotype, as they express cytokeratins 5/14, p63, and integrin $\alpha 6$ (11). There is also evidence, however, that the luminal compartment may contain stem/progenitor cells and that these give rise to basal cells.

Experiments involving labeling cells with the synthetic nucleoside bromo-deoxy-uridine to detect those that are proliferating indicate that slow-cycling stem cells are concentrated in the proximal region of prostatic ducts adjacent to the urethra and that both basal and luminal compartments contain slow-cycling cells. Cells from this region have substantial growth potential in vivo and in vitro and can be serially passaged in vivo at least four times. It is not known whether CARNs are concentrated in the proximal region, but if so, CARNs may comprise some of the slow-cycling proximal luminal cell population.

These results provide a possible means to address the CSC signature issue. However, it is not clear that the result is definitive nor of immediate clinical use.

Stem cells are known in hematopoietic cell generation. They are isolated, separate and their ability to develop the full plethora of blood cells is well known. The stem cell concept applied to say prostate cells or skin cells is of more recent structure and is in many ways still open for debate. Taking that construct one step further and considering a cancer stem cell is possible even more of a conjecture. We can accept the concept of a cancer stem cell in the many blood cancers. We know that CML may very well have a translocation, as is found in other leukemias. Yet the establishment of the same for say prostate and melanoma malignancies is I believe still a work in progress.

For example as Jeet et al state:

Different stages of prostate cancer progression: (a) prostatic intraepithelial neoplasia, a premalignant lesion considered to be a precursor to invasive carcinoma; (b) primary localized adenocarcinoma, dependent on androgen stimulus and can be treated by androgen ablation; (c) androgen-independent prostate cancer, tumor then becomes androgen independent and metastasizes to other organs (e.g., lung, bone, and lymph node)

The linear progression we have disputed in prior writings based upon clinical observations. The reason is that we have observed the remission of diffuse HGPIN in patients at first biopsy and then the absence in subsequent. Not just reduction of HGPIN, but total elimination. Our hypothesis is that there has been the presence of a stem cell and its removal during the first extensive core biopsy, usually 16 or more cores, not classic sextant biopsy.

Stem cells are a powerful paradigm which may very well align with the clonal model. For if it is the stem cell which has suffered the genetic change then if this cell has the controlling powers attributed to it, then the stem cell model will also tell us a great deal regarding treatment, and our inability to do so.

For example, a stem cell will itself generate other stem cells as well as non-stem cells.

7.5 SUMMARY AND ISSUES

There are many questions still posed regarding the cancer stem cell:

1. What are the pathway dynamics and are they the same in the non-stem like cells?
2. What is the driver for the kinetics of a CSC? Namely do we have a dramatically different set of kinetics?
3. What is the mechanism for the progression of subsequent mutations in a CSC?
4. How do we identify the CSC in a sample biopsy? Are there specific cell markers and are they consistent or do they change?
5. What are the driving ligands which activate a CSC?
6. Do stem cells have true pluripotency or are they cell specific?
7. What are the stem cell surface ligands and receptors which promote mitosis and how are they transmitted across a group of cells?
8. What causes a stem cell, specifically a CSC, to evolve and how does that occur?

We can continue with a great number of these types of questions. However if one hopes to be able to model cancer pathway dynamics one must first address the issue of the CSC, for if the CSC has the definitive characteristics that we have discussed then it and it alone is what should be focused upon. Furthermore the examination of cells for pathway markers may very well have to be done only on the CSC, which then argues that we need sophisticated techniques to identify them and extract them as well.

8 EPIGENETIC FACTORS

Epigenetic modifications are considered heritable changes in gene expression occurring when there is no true underlying change in the DNA⁸⁰. Epigenetic silencing of tumor suppressor genes is one of the most significant contributors to cancer development. Recent summaries by Esteller provides 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.

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.

8.1 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.

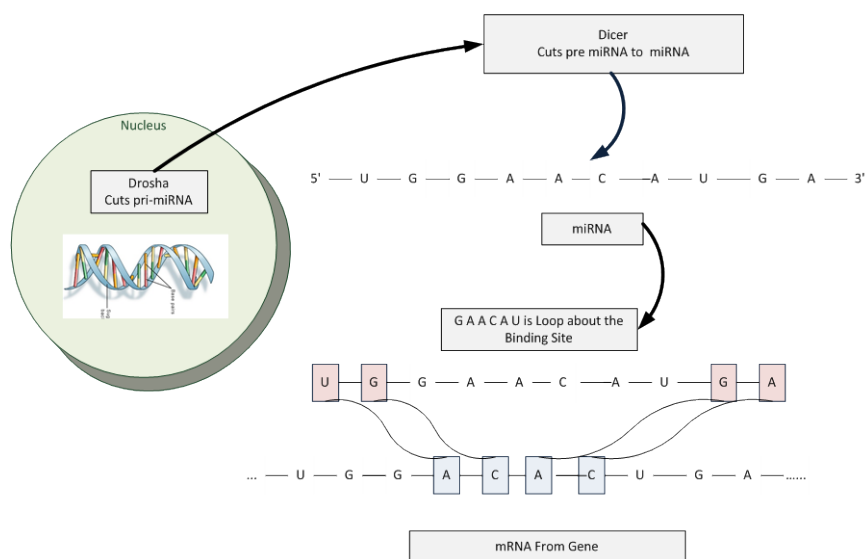
We now briefly examine the miRNA production and action. This is graphically demonstrated below. From segments of the DNA, segments not containing genes, a long segment called a pri-miRNA is generated and it is then cut to a shorter segment called a pre-miRNA and transported

⁸⁰ See Pali and Robertson (2007)

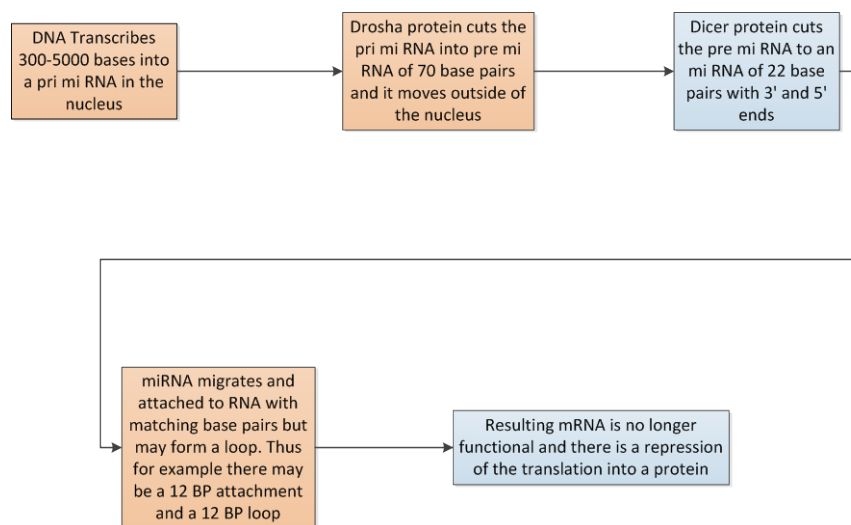
to the cytoplasm outside of the nucleus. Then another protein called Dicer cuts up the pre miRNA into about 22 base single-stranded pair segments which are the miRNA,

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.

8.2 siRNA AND MIRNA

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.

8.3 DYNAMICS OF MIRNA

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 is 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

$[Pro] = \text{Concentration of related promoter}$

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

$$\frac{d[mRNA_i]}{dt} = K_{m,i}[Pro_{m,i}] - \sum_{n=1}^N \kappa_{i,n}[miRNA_{i,n}]$$

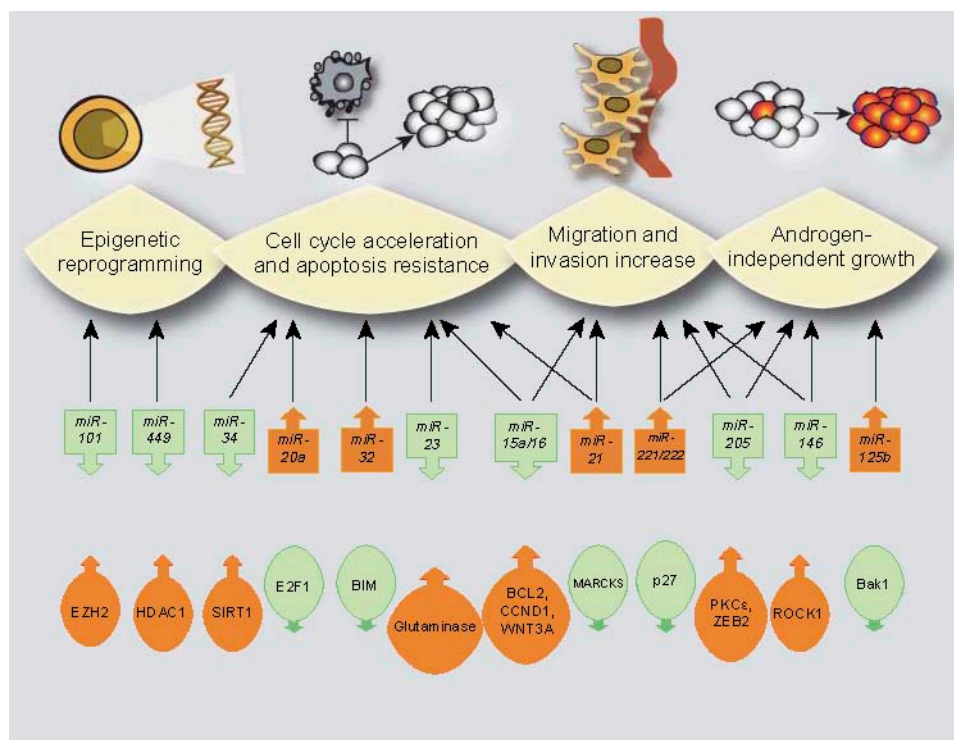
where

$[Pro] = \text{Concentration of related promoter}$

8.4 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.

The graphic from Coppola et al is shown below where it depicts a collection of miRNAs which impact various parts of the PCa process.



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 have 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, to X-chromosome inactivation in mammals. 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 level.

*Members of the miRNA family were initially discovered as small temporal RNAs (stRNAs) that regulate developmental transitions in **Caenorhabditis elegans**. 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 leukaemia³⁰, 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 tumours³¹ (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.

8.5 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.

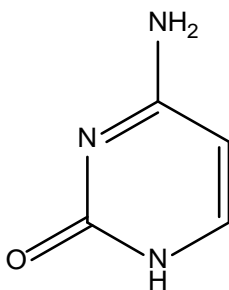
Inhibition of Wnt signalling may prove to be an effective road to inhibit the uncontrolled cell renewal that drives cancer. Acting as novel and pivotal regulators of protein-encoding genes, miRNAs will have great potential in regulating CSCs’ biological functions by targeting CSCs-related signal pathway 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.

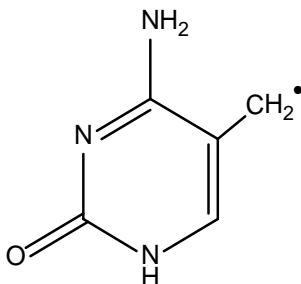
8.6 METHYLATION

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 Zuberan. 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.

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 5^o 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, DNMT3a, and DNMT3b. DNA methylation 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 Miranda 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.

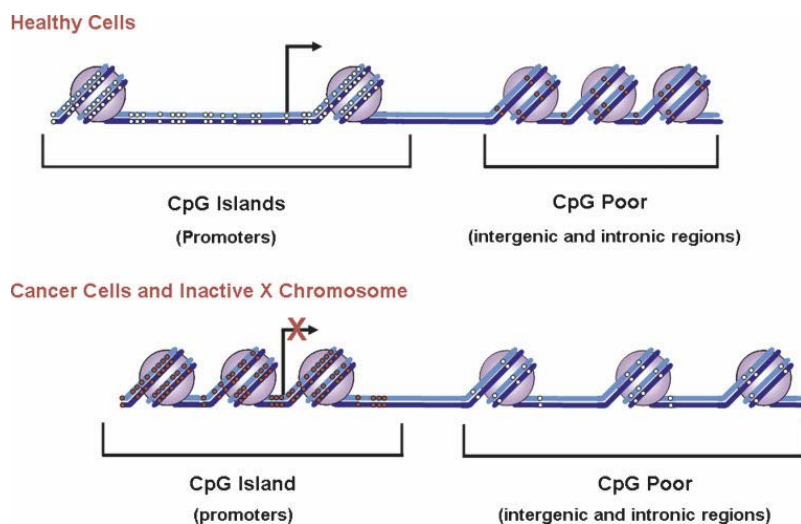
When found within promoters, DNA methylation prevents the reactivation of silent genes, even when the repressive histone marks are reversed. This allows the daughter cells to retain the same

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:

| <i>Gene</i> | <i>Function</i> |
|-------------------|--|
| 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.

9 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 leading to PCa.

9.1 EXAMPLE REACTION

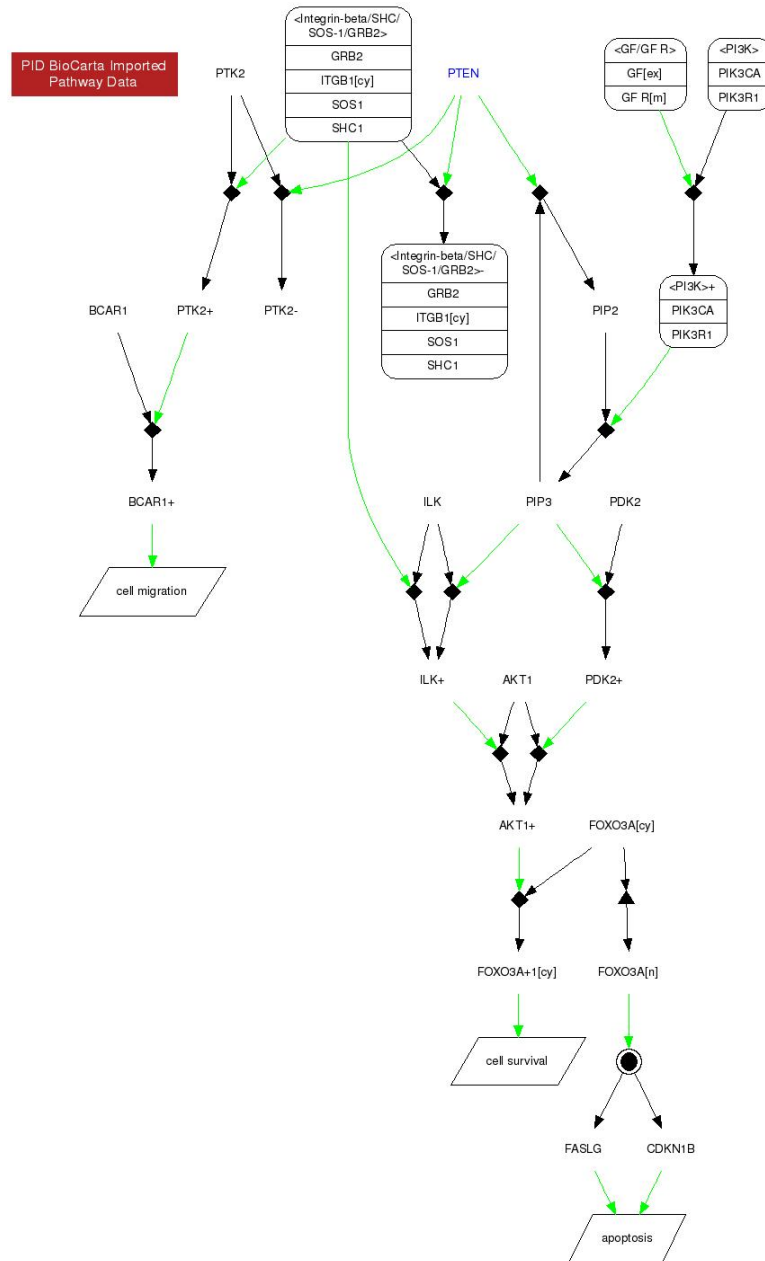
Let us begin with a simple example, the PTEN reaction. We will examine several varying descriptions and then focus on a specific model. The issues here will be fundamentally the following:

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 PCa 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 mTOR⁸¹. This version is a quite complex pathway.

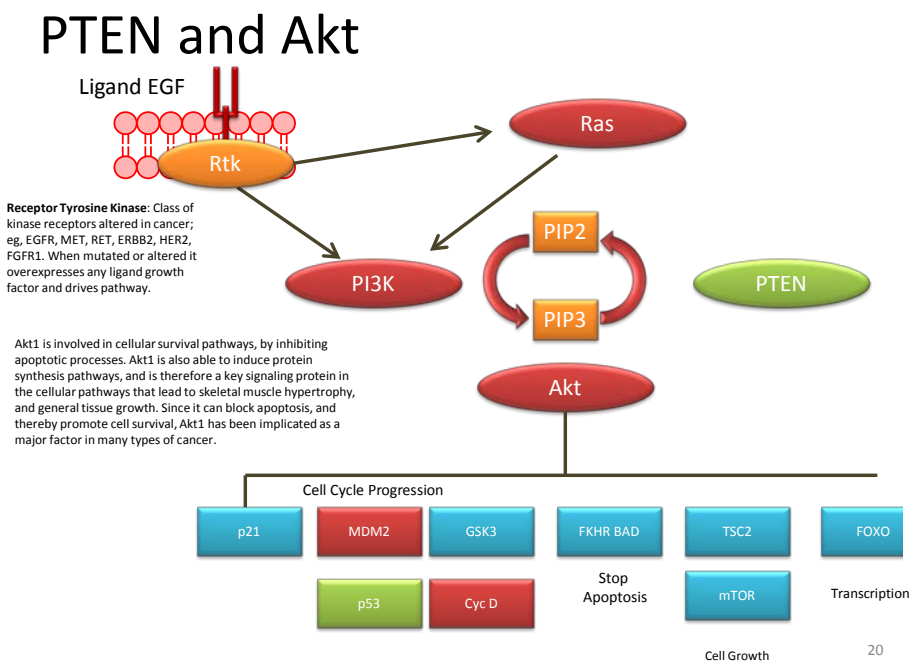
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http://pid.nci.nih.gov/search/pathway_landing.shtml?what=graphic&jpg=on&pathway_id=100101&source=2&output-format=graphic&ppage=1&genes_a=5728

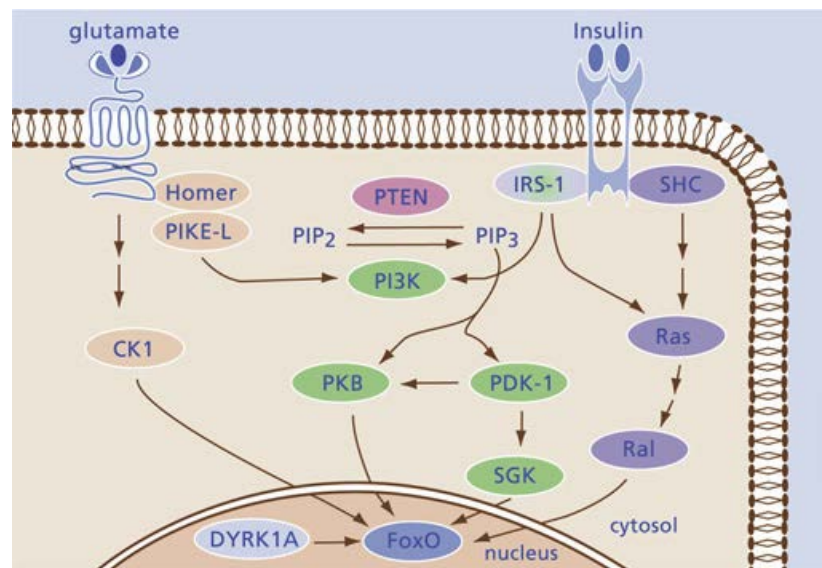


Each step is a reaction and each reaction is facilitated by some enzyme, other protein, which leads to the end result. However since the enzymes are in time varying concentrations and the reactions are temporal and dependent on the specific concentrations, we have a definable but complex dynamic system.

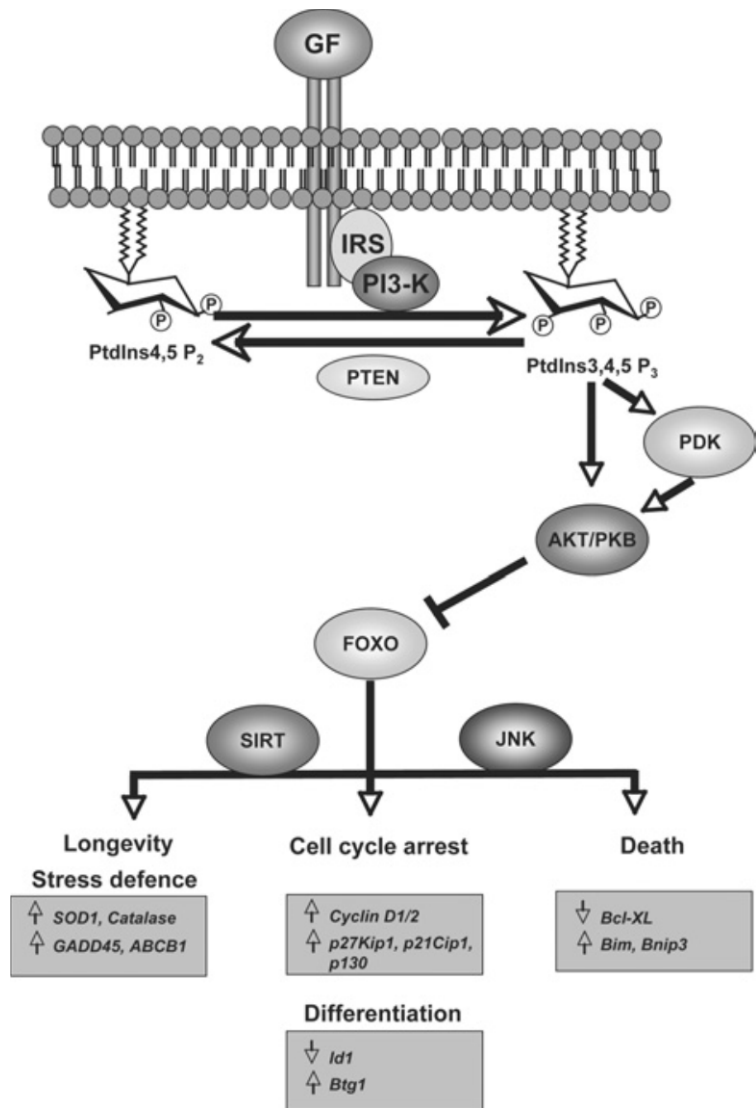
The above reaction can be simplified as follows:



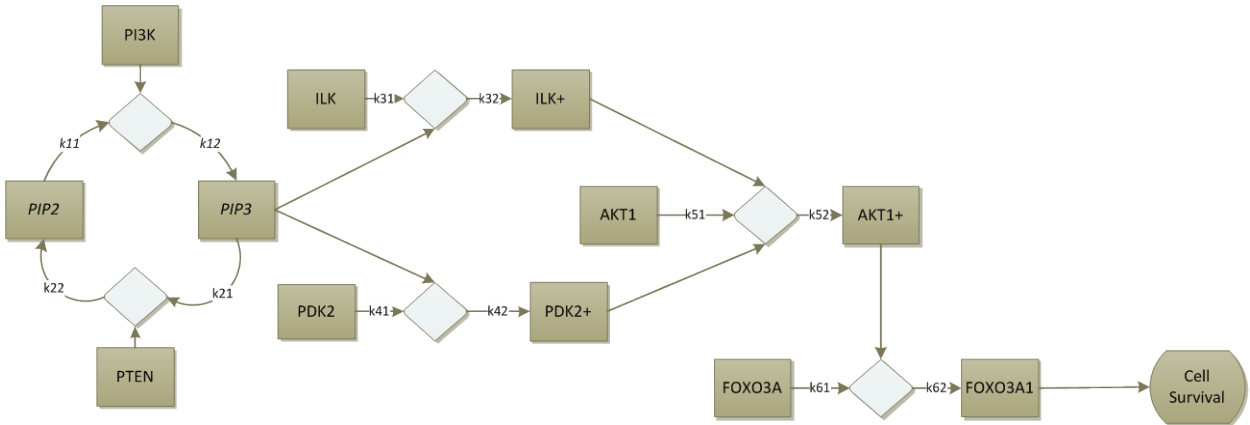
Another view of the pathway is by van der Heidde et al;



Also there is another view in Lam et al:



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:

$$\begin{aligned}\frac{d[PIP2]}{dt} &= v_{12} - v_{21} \\ \frac{d[ILK]}{dt} &= v_{31} \\ \frac{d[ILK+]}{dt} &= v_{13} \\ \frac{d[PDK2]}{dt} &= v_{41} \\ \frac{d[PDK2+]}{dt} &= v_{14} \\ \frac{d[AKT1]}{dt} &= v_{51} \\ \frac{d[AKT+]}{dt} &= v_{15} \\ \frac{d[FOXO3A]}{dt} &= v_{61} \\ \frac{d[FOXO3A1]}{dt} &= v_{16} \\ \frac{d[Cell\ Survival]}{dt} &= v_7\end{aligned}$$

The reaction rates, v , are in several cases determined by the enzymatic concentrations of the prior reactions such as:

$$\begin{aligned}v_{21} &= k_{21}[PTEN] \\ v_{12} &= k_{12}[PI3K] \\ v_{42} &= k_{42}[PIP3] \\ v_{51} &= k_{51}[ILK+] + k_{52}[PDK2+] \\ v_{61} &= k_{61}[AKT1+]\end{aligned}$$

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.

9.2 PCA REACTION MODELS: A SUBSET

9.3 HG PIN AND GSTP1

PID BioCarta Imported Pathway Data

The diagram illustrates the metabolic pathways for the detoxification of xenobiotics. It features several interconnected nodes and reactions:

- Alkylating xenobiotics** and **gsh** (glutathione) are converted into **GS-conjugates[cy]** (cytosolic GS-conjugates) via a reaction involving **GSTP1** (glutathione S-transferase P1).
- GS-conjugates[cy]** are then converted into **GS-conjugates[ex]** (extracellular GS-conjugates) via a reaction involving **ABCC1[m]** (ATP-binding cassette, class C, member 1, membrane).
- ABCC3[m]** (ATP-binding cassette, class C, member 3, membrane) is involved in the transport of **Bile salts[ex]** (extracellular bile salts) into the cell, where they are converted into **Bile salts[cy]** (cytosolic bile salts).
- ABCB11[m]** (ATP-binding cassette, class B, member 11, membrane) is involved in the transport of **Hydrophobic compounds[ex]** (extracellular hydrophobic compounds) into the cell, where they are converted into **Hydrophobic compounds[cy]** (cytosolic hydrophobic compounds).
- ABCB1[m]** (ATP-binding cassette, class B, member 1, membrane) is involved in the transport of **Hydrophobic compounds[ex]** (extracellular hydrophobic compounds) into the cell, where they are converted into **Hydrophobic compounds[cy]** (cytosolic hydrophobic compounds).
- lecithin[ex]** (extracellular lecithin) is converted into **lecithin[cy]** (cytosolic lecithin) via a reaction involving **ABCB4[m]** (ATP-binding cassette, class B, member 4, membrane).
- GS-conjugates[cy]** are also converted into **lecithin[cy]** (cytosolic lecithin) via a reaction involving **ABCB4[m]** (ATP-binding cassette, class B, member 4, membrane).

9.4 TOTAL PATHWAYS

340 | Page

In addition the MSKCC

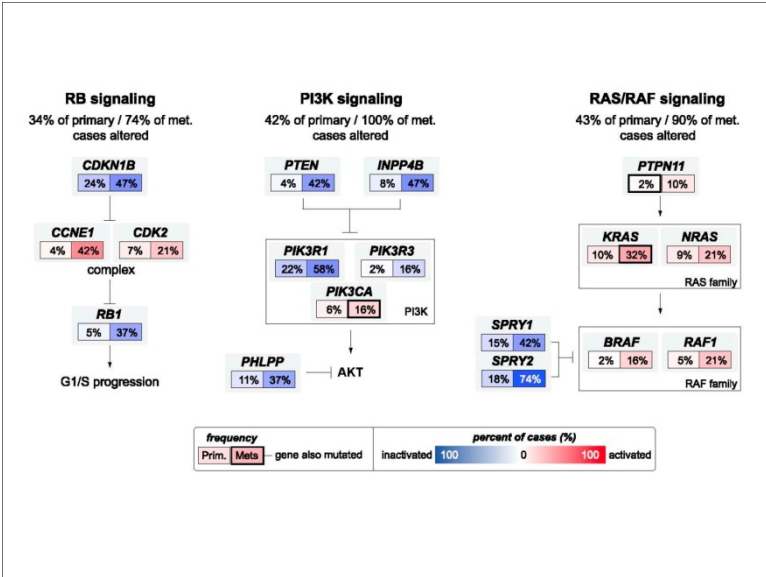
Specific Pathways

Pathways involved in prostate cancer



9.5 SPECIFIC PATHWAYS

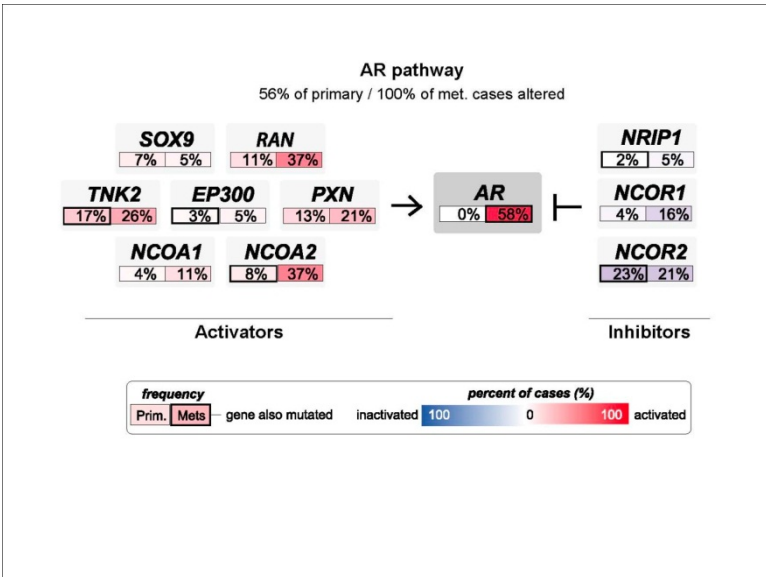
In addition the MSKCC site presents details on specific pathways as relates to PCa. We present them as follows. First we depict the major gene and gene products and then show what percent of the PCa cells exhibit modifications of those specific genes. For example in the PTEN related pathway, 42% of the PCa mets exhibit PTEN loss and in met PCa we have 100% of the cells exhibiting some loss in some element of the pathway. BRAF is activated in 16% of mets, KRAS in 32%. CDKN1B is repressed in 47% of mets.



The immediate conclusion from the above is that there is no clear pattern of what is repressed or activated. There is not a clear and unambiguous pattern of steps which define PCa or met PCa.

In a sense this lack of intensity of any one or a set of genes should be a concern. It infers that there very well may be multiple pathways for the development of PCa and mets related thereto.

The following shows the results for the AR pathway. Here we see that the AR is overexpressed in 58% of met PCa.



9.6 REACTION KINETICS

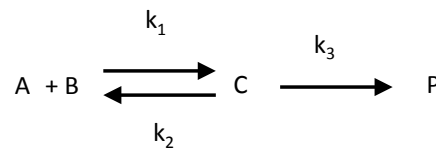
Reaction kinetics is a powerful set of chemical dynamics that make the cell function. In this section we review several of the models for reaction kinetics including the Michaelis-Menten model⁸⁴. To start we know that there is a change in a concentration C of some substance and that the concentration may be resulting from one or several competing processes. The following is a general statement of that result;

$$\frac{dC}{dt} = [Synthesis] - [Degradation] - [Phosphorylation] \\ + [Dephos] - [Binding] + [Release] + etc$$

9.6.1 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.

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:

$$K = \frac{[C]}{[A][B]}; \quad \text{and} \quad K = \frac{k_1}{k_2}$$

$$\frac{d[P]}{dt} = k_3 [C] = k_3 K [A][B] = k [A][B] \quad \text{where} \quad k = \frac{k_1 k_3}{k_2}$$

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.

⁸⁴ See p. 111 Murray.

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 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 follows:



We first note that this reaction is what controls the production of an anthocyanin in the secondary 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.

Let us take a simple example from botany, namely plant color. Plant colors result from the concentrations of a set of anthocyanins. The concentration of each type in a mix will result in what we see, and seeing a color one may invert the result and attempt to ascertain the anthocyanins present. What drives the production of anthocyanins is the presence of enzymes,

ones for each anthocyanin path. The enzymes are the direct protein products of the plants gene. Thus, we need to understand this reaction since when we look at plant colors we are effectively looking at anthocyanin concentrations, and more importantly the target protein, enzyme or gene productions controlling the secondary pathways. If we were to focus on say the protein E, then the more E the more P. Conversely the less E the less P. This domain is where we must look. In most enzyme reactions we have always assumed that E was excessive. That E could not be exhausted and that given the available E the reaction as above proceeds. This may not be the case here, thus the range of evaluating the solution must be somewhat expansive.

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 follows⁸⁵:

$$\frac{ds}{dt} = -k_1 es + k_{-1} c$$

$$\frac{de}{dt} = -k_1 es + (k_{-1} + k_2) c$$

$$\frac{dc}{dt} = k_1 es - (k_{-1} + k_2) c$$

$$\frac{dp}{dt} = k_2 c$$

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 calculated if $c(t)$ has been calculated

2. If we add the equations for e and c we find that:

$$\frac{de(t)}{dt} + \frac{dc(t)}{dt} = 0$$

⁸⁵ See Murray p. 310.

Thus we are left with two differential equations:

$$\begin{aligned}\frac{ds}{dt} &= -k_1s + (k_1 + k_{-1})c \\ \frac{dc}{dt} &= k_1e_0s - (k_1 + k_{-1} + k_2)c\end{aligned}$$

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:

$$\begin{aligned}\frac{ds}{dt} &= -k_1s + (k_1 + k_{-1})c \\ \frac{de}{dt} &= -k_1e_0s + (k_1 + k_{-1} + k_2)c\end{aligned}$$

and then solve for s and e . Now for the normalizations we define:

$$\begin{aligned}\tau = k_1e_0t, u(\tau) &= \frac{s(t)}{s_0}, v(\tau) = \frac{c(t)}{c_0} \\ \lambda = \frac{k_2}{k_1s_0}, K = \frac{k_{-1} + k_2}{k_1s_0}, \varepsilon &= \frac{e_0}{s_0}\end{aligned}$$

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:

$$\begin{aligned}\frac{du}{d\tau} &= -u + (u + K - \lambda)v \\ \varepsilon \frac{dv}{d\tau} &= u - (u + K)v \\ u(0) &= 1 \\ v(0) &= 0\end{aligned}$$

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.

$$K = \frac{[C]}{[A][B]},$$

$$\text{and } K = \frac{k_1}{k_2}$$

$$\frac{d[P]}{dt} = k_3[C] = k_3K[A][B] = k[A][B] \text{ where } k = \frac{k_1k_3}{k_2}$$

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 anthocyanin The ability to apply to multiple anthocyanin 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, an anthocyanin. The more of that protein we have, the greater its concentration, the more secondary product we can get. This is P is the controlling protein concentration, we have:

$$\frac{\partial P_n(x, t)}{\partial t} = H(P_n, A_n, R_n, x, t) + D_P \nabla^2 P_n(x, t)$$

We show such pathways below. In the above pathway we have a complex but modellable set of interactions. They are characterized by:

When there are multiple A-R interactions then they add and the net result is an overlapping of the anthocyanin pathway products. The overlays can be shown to create the typical patterns 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 tempo-spatial dynamics of the concentrations of the products of the activator and repressor genes, A and S respectively. We assume we have a model as shown below:

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.

9.6.3 Basic Kinetics and Reactions

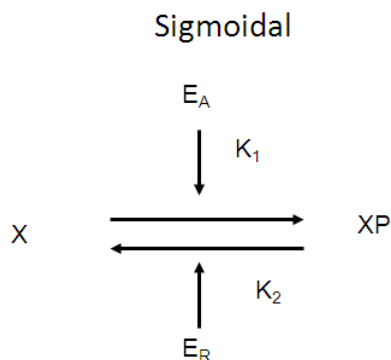
We can now look at a general set of reactions. We assume some reactant X with a concentration [X] and they concentration may increase by means of several different processes. We provide a generic example which is hardly inclusive:

$$\frac{d[X]}{dt} = [Synthesis] - [Degradation] - [Phosphorylation] + [Dephosphorylation] - [Binding] + [Release] + etc$$

Now we want to describe a set of such models for the subsequent analysis. Understanding each provides a basis for understanding an integral whole.

9.6.4 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 phosphorylation-dephosphorylation model or the sigmoidal model⁸⁶. 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.



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.

⁸⁶ There are a multitude of models here.

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

$$g([X]) = \frac{k_1[X]}{K_{m1} + [X]}$$

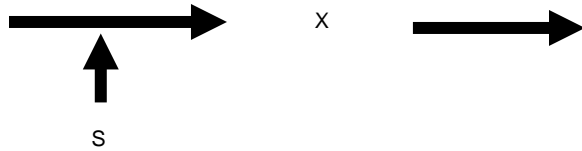
and

$$h([X]) = \frac{k_2(x_T - [X])}{K_{m2} + x_T - [X]}$$

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.

9.6.5 Synthesis and Degradation

This is an example from Szalasi 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.

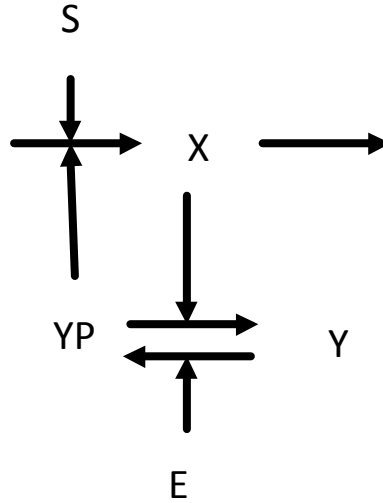
$$\frac{d[X]}{dt} = k_1[S] - k_2[X]$$

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.

9.6.6 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,

Negative Feedback



The model then is as follows:

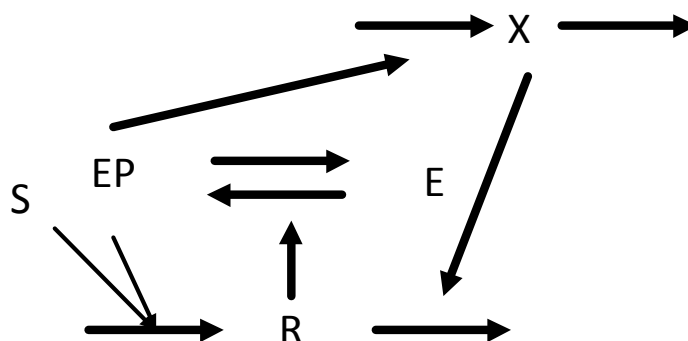
$$\frac{d[X]}{dt} = k_1[S] - k_2[YP][X]$$

$$\frac{d[YP]}{dt} = \frac{k_3[x][x](y_T - [YP])}{K_{m3} + y_T - [YP]} - \frac{k_4[E][YP]}{K_{m4} + [YP]}$$

9.6.7 Activator Inhibitor

This process has an internal process which activate 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:

$$\frac{d[R]}{dt} = k_0[EP] + k_1[S] - k_2[X][R]$$

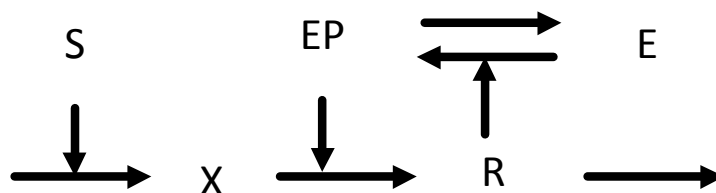
$$\frac{d[X]}{dt} = k_3[EP] - k_4[X]$$

Like the other complex processes this shows cyclic instability.

9.6.8 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:

$$\frac{d[X]}{dt} = k_1[S] - (k'_0 + k_0[EP])[X]$$
$$\frac{d[R]}{dt} = (k'_0 + k_0[EP])[X] - k_2[R]$$

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.

10 PROGNOSTIC MARKERS

We have examined many studies looking at genetic prognostic markers from a causative basis. Namely we look at genes in specific pathways which are altered result in malignant conditions for cell growth and proliferation. In this note we look at a recent article examining blood borne proteins which have some putative prognostic value.

Both reports we discuss herein are prognostic in their approach. They are prognostic, however, for androgen resistant PCa. Although it is always good to understand what the prognosis is, even if you cannot do anything about it, it does raise the concern of what benefit is this to either the physician or patient. The results seem to say that the prognosis is that one has 9-10 months versus 3-4 years of expected life. There is nothing that can be done, and even care of the patient is in question. No matter what it is palliative. Although the results are interesting the question is are they beneficial, to anyone. One may ask why waste the money to find out something that you can do nothing about. That is both an ethical and an economic issue.

A summary reported in the press states⁸⁷:

The first study, demonstrating that a nine-gene signature could distinguish between lower and higher risk castration-resistant prostate cancer (CRPC), was led by Johann de Bono, MD, of the drug development unit at the Royal Marsden NHS Foundation Trust in Sutton, United Kingdom, and was conducted with colleagues in both Europe and the United States.

The second study, which found a six-gene signature that also stratified CRPC into different risk groups, was led by William K. Oh, MD, of the division of hematology/oncology at the Tisch Cancer Institute at Mount Sinai School of Medicine in New York.

One of the findings was related to immune system genes not those normally thought of in pathway control. The authors state⁸⁸:

The result that immune function is key to prostate cancer outcome is very surprising, said Dr. de Bono.

“The biggest surprise of this study was that the most significant six genes which predicted survival were not primarily cancer-related genes, but were involved in immune function,” said Dr. Oh. “In some ways, this is not a surprise, since it suggests that the patient’s innate immune response to cancer may be a strong predictor of the impact of the cancer.” Dr. Oh added that the function of the identified genes in the immune system is not yet understood. Nor is it understood how the genes may interact and lead to a difference in survival for patients.

Both authors of the studies see the RNA analysis as highly applicable for the clinic. Dr. Oh said that the particular six-gene signature his study identified “could be translated fairly easily to the

⁸⁷ <http://www.cancernetwork.com/prostate-cancer/content/article/10165/2106848>

⁸⁸ <http://www.cancernetwork.com/prostate-cancer/content/article/10165/2106848>

clinic, since it uses simple technologies such as PCR to identify the genes of interest.” Dr. Oh and colleagues collected the RNA in blood using a special preservation tube (PAXgene), which are widely available. Dr. de Bono and colleagues are currently testing whether a DNA analysis could provide the same information.

Dr. Oh highlighted the different approaches of the two teams: “What is interesting about the Royal Marsden paper is that they took a very different analytic approach, which in fact looked at more genes and was thus potentially more unbiased, and found that the most prognostic genes were again driving immune function in patients.” Both teams ended up with a similar result: “The blood contains a molecular signature in patients with advanced prostate cancer which predicts survival based on the functioning of the immune system.”

Now it must be emphasized that these studies examined prognostic factors and not diagnostic and that further they examined patients who were already androgen resistant, namely the PCa had progressed extensively. Thus the implication of immune system elements is not unexpected. Also this analysis is not diagnostic in any way and further is not prognostic in any manner related to a watchful waiting strategy. As the authors suggest survival in his risk is about 8 months and in “low” risk is about 35 months. In either case the patient is terminal.

10.1 RECENT FINDING

There are two recent papers regarding this issue. The first is a recent Lancet article by Ross et al, entitled, *A whole-blood RNA transcript-based prognostic model in men with castration-resistant prostate cancer: a prospective study*⁸⁹, the authors state:

Survival for patients with castration-resistant prostate cancer is highly variable. We assessed the effectiveness of a whole-blood RNA transcript-based model as a prognostic biomarker in castration-resistant prostate cancer. Peripheral blood was prospectively collected from 62 men with castration-resistant prostate cancer on various treatment regimens ...

A six-gene model (consisting of ABL2, SEMA4D, ITGAL, and C1QA, TIMP1, CDKN1A) separated patients with castration-resistant prostate cancer into two risk groups: a low-risk group with a median survival of more than 34.9 months (median survival was not reached) and a high-risk group with a median survival of 7.8 months (95% CI 1.8–13.9; $p < 0.0001$). The prognostic utility of the six-gene model was validated in an independent cohort. This model was associated with a significantly higher area under the curve compared with a clinicopathological model (0.90 [95% CI 0.78–0.96] vs 0.65 [0.52–0.78]; $p = 0.0067$).

Transcriptional profiling of whole blood yields crucial prognostic information about men with castration-resistant prostate cancer. The six-gene model suggests possible dysregulation of the immune system, a finding that warrants further study.

We wish to examine this in some detail. There are several issues we wish to look at.

⁸⁹ http://www.thelancet.com/journals/lanonc/article/PIIS1470-2045%2812%2970263-2/fulltext?_eventId=login

First, what pathways do these genes participate in and thus how do they play a role in the management of the homeostasis of the cell. Why would one want to consider these genes?

Second, are these genes causative or reflective of a cancer state? If reflective are there causative genes related thereto which may merit more detailed examination.

Third from a prognostic perspective, why are these expressed as they are?

Fourth from a treatment perspective are these markers useful in targeting gene aberrations so as to mitigate further uncontrolled growth and in fact reduce what is present.

Fifth, is there a holistic picture of how most likely metastatic growth is identified by such expression and how one may ascertain the spread of the metastatic cells?

There is also a second paper entitled, *Prognostic value of blood mRNA expression signatures in castration-resistant prostate cancer: a prospective, two-stage study* by Olmos et al which notes⁹⁰:

Biomarkers are urgently needed to dissect the heterogeneity of prostate cancer between patients to improve treatment and accelerate drug development. We analysed blood mRNA expression arrays to identify patients with metastatic castration-resistant prostate cancer with poorer outcome.

Whole blood was collected into PAXgene tubes from patients with castration-resistant prostate cancer and patients with prostate cancer selected for active surveillance. In stage I (derivation set), patients with castration-resistant prostate cancer were used as cases and patients under active surveillance were used as controls. These patients were recruited from The Royal Marsden Hospital NHS Foundation Trust (Sutton, UK) and The Beatson West of Scotland Cancer Centre (Glasgow, UK).

In stage II (validation-set), patients with castration-resistant prostate cancer recruited from the Memorial Sloan-Kettering Cancer Center (New York, USA) were assessed. Whole-blood RNA was hybridised to Affymetrix U133plus2 microarrays. Expression profiles were analysed with Bayesian latent process decomposition (LPD) to identify RNA expression profiles associated with castration-resistant prostate cancer subgroups; these profiles were then confirmed by quantitative reverse transcriptase (qRT) PCR studies and correlated with overall survival in both the test-set and validation-set.

LPD analyses of the mRNA expression data divided the evaluable patients in stage I (n=94) into four groups. All patients in LPD1 (14 of 14) and most in LPD2 (17 of 18) had castration-resistant prostate cancer. Patients with castration-resistant prostate cancer and those under active surveillance comprised LPD3 (15 of 31 castration-resistant prostate cancer) and LDP4 (12 of 21 castration-resistant prostate cancer).

⁹⁰ <http://www.thelancet.com/journals/lanonc/article/PIIS1470-2045%2812%2970372-8/fulltext>

Patients with castration-resistant prostate cancer in the LPD1 subgroup had features associated with worse prognosis and poorer overall survival than patients with castration-resistant prostate cancer in other LPD subgroups (LPD1 overall survival 10·7 months [95% CI 4·1—17·2] vs non-LPD1 25·6 months [18·0—33·4]; $p<0\cdot0001$).

A nine-gene signature verified by qRT-PCR classified patients into this LPD1 subgroup with a very low percentage of misclassification (1·2%). The ten patients who were initially unclassifiable by the LPD analyses were subclassified by this signature. We confirmed the prognostic utility of this nine-gene signature in the validation castration-resistant prostate cancer cohort, where LPD1 membership was also associated with worse overall survival (LPD1 9·2 months [95% CI 2·1—16·4] vs non-LPD1 21·6 months [7·5—35·6]; $p=0\cdot001$), and remained an independent prognostic factor in multivariable analyses for both cohorts.

Our results suggest that whole-blood gene profiling could identify gene-expression signatures that stratify patients with castration-resistant prostate cancer into distinct prognostic groups.

10.2 SUMMARY OF PROGNOSTIC GENE MARKERS

The following Table is a summary of the prognostic gene markers.

| <i>Gene</i> | <i>Description</i> ⁹¹ | <i>Location</i> |
|---------------|--|-----------------|
| <i>ABL2</i> | This gene encodes a member of the Abelson family of nonreceptor tyrosine protein kinases. The protein is highly similar to the c-abl oncogene 1 protein, including the tyrosine kinase, SH2 and SH3 domains, and it plays a role in cytoskeletal rearrangements through its C-terminal F-actin- and microtubule-binding sequences. This gene is expressed in both normal and tumor cells, and is involved in translocation with the ets variant 6 gene in leukemia. Multiple alternatively spliced transcript variants encoding different protein isoforms have been found for this gene. | <i>1q25.2</i> |
| <i>SEMA4D</i> | CD100; SEMAJ; coll-4; C9orf164; M-sema-G. Semaphorin 4D (Sema 4D) is an axon guidance molecule which is secreted by oligodendrocytes and induces growth cone collapse in the central nervous system. By binding plexin B1 receptor it functions as an R-Ras GTPase-activating protein (GAP) and repels axon growth cones in both the mature central nervous system. In the immune system, CD100 binds CD72 to activate B cells and dendritic cells, though much about this interaction is still under investigation. During skin damage repairs, SEMA4D interacts with Plexin B2 on gamma delta T cells to play a role in the healing process | <i>9q22.2</i> |
| <i>ITGAL</i> | ITGAL encodes the integrin alpha L chain. Integrins are heterodimeric integral membrane proteins composed of an alpha chain and a beta chain. This I-domain containing alpha integrin combines with the beta 2 chains (ITGB2) to form the integrin lymphocyte function-associated antigen-1 (LFA-1), which is expressed on all leukocytes. LFA-1 plays a central role in leukocyte intercellular adhesion through interactions with its ligands, ICAMs 1-3 (intercellular adhesion molecules 1 through 3), and also functions in lymphocyte costimulatory signaling. Two transcript variants encoding different isoforms have been found for this gene. | <i>16p11.2</i> |
| <i>C1QA</i> | This gene encodes a major constituent of the human complement subcomponent C1q. C1q associates with C1r and C1s in order to yield the first component of the serum complement system. Deficiency of C1q has been associated with lupus erythematosus and glomerulonephritis. C1q is composed of 18 polypeptide chains: six A-chains, six B-chains, and six C-chains. Each chain contains a collagen-like region located near the N terminus and a C-terminal globular region. The A-, B-, and C-chains are arranged in the order A-C-B on chromosome 1. This gene encodes the A-chain polypeptide of human complement subcomponent C1q. | <i>1p36.12</i> |
| <i>TIMP1</i> | This gene belongs to the TIMP gene family. The proteins encoded by this gene family are natural inhibitors of the matrix metalloproteinases (MMPs), a group of peptidases involved in degradation of the extracellular matrix. In addition to its inhibitory role against most of the known MMPs, the encoded protein is able to promote cell proliferation in a wide range of cell types, and may also have an anti-apoptotic function. Transcription of this gene is highly inducible in response to many cytokines and hormones. In addition, the expression from some but not all inactive X chromosomes suggests that this gene inactivation is polymorphic in human females. This gene is located within intron 6 of the synapsin I gene and is transcribed in the opposite direction. | <i>Xp11.3</i> |
| <i>CDKN1A</i> | This gene encodes a potent cyclin-dependent kinase inhibitor. The encoded protein binds to and inhibits the activity of cyclin-CDK2 or -CDK4 complexes, | <i>6q21.2</i> |

⁹¹ <http://www.ncbi.nlm.nih.gov/gene>

| <i>Gene</i> | <i>Description</i> ⁹¹ | <i>Location</i> |
|-------------|--|-----------------|
| | 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. | |

10.3 TIMP-1

TIMP-1 is a tissue inhibitor of metalloproteinases. Metalloproteinases (matrix metalloproteinases, MMP) are zinc dependent proteases which have the ability to cleave cell walls⁹². Transcription of this gene and thus increase in its product are activated by cytokines and various hormones. However, in this analysis, it is most likely the excitation from the immune system cytokines which activate the response.

There has been extensive work performed analyzing TIMP-1 recently in various other cancers. The work of Wang et al examines Gastric cancers, Lee examines Colorectal cancers, and Bloomston looks at pancreatic cancers. Other detailed analyses have been done by Vaghooti et al as well as Wang. Thus it should be no surprise as to the use of TIMP-1 in this specific case as well.

In addition as per Marks et al⁹³, The TIMP, tissue inhibitors of metalloproteinases, MMP, are within the class of ADAM proteins which are membrane bound.

The following is a summary by Bigelow et al and although it focuses on breast cancer issues it does provide a reasonable summary as applied to this case:

TIMP-1 (Tissue inhibitor of matrix metalloproteinase-1) is typically associated with inhibition of matrix metalloproteinases (MMP) induced invasion. However, TIMP-1 is overexpressed in many malignancies and is associated with poor prognosis in breast cancer.

The mechanisms by which TIMP-1 promotes tumorigenesis are unclear. Reduced levels of TIMP-1 mediated by shRNA in MDA-MB-231 breast cancer cells had no effect on cellular physiology in vitro or tumor growth in SCID mice compared to vector control MDA-MB-231 cells.

However, overexpression of TIMP-1 in MDA-MB-231 cells resulted in inhibition of cell invasion and enhanced phosphorylation of p38 MAPK and AKT in vitro. Additionally, treatment of parental MDA-MB-231 cells with purified TIMP-1 protein led to activation of p38 MAPK and MKK 3/6. cDNA array analysis demonstrated that high expression of TIMP-1 in MDA-MB-231 cells resulted in alterations in expression of approximately 200 genes, 1.5 fold or greater compared to vector control cells ($P < 0.1$).

Real-time RT-PCR confirmed changes in expression of several genes associated with cancer progression including DAPK1, FGFR4 and MAPK13.

In vivo, high TIMP-1 expression induced tumor growth in SCID mice compared to vector control cells and increased tumor vessel density. Affymetrix array analysis of vector control and TIMP-1

⁹² For example the use of doxycycline as a suppressor of MMP at low doses is used to treat corneal abrasions and certain types of dental erosions.

⁹³ See Marks et al, pp. 455-459.

MDA-MB-231 xenograft tumors revealed that TIMP-1 altered expression of approximately 600 genes in vivo, including MMP1, MMP13, S100A14, S100P, Rab25 and ID4.

These combined observations suggest that the effects of TIMP-1 differ significantly in a 2-D environment compared to the 3-D environment and that TIMP-1 stimulates tumor growth.⁹⁴

Thus we have the question that TIMP-1 at an inhibitor of MMP is thus increased in response to cytokines which may themselves be increased as a result of the PCa metastatic expansion. The question then becomes; is this just a natural and expected result, is this just consistent with PCa evolution, or is there something special here.

10.4 ABL2

BCR and ABL are genes closely related to CML. In a 2002 paper in NEJM by Katarjian et al we have:

Chronic myelogenous leukemia (CML) accounts for about 20 percent of newly diagnosed cases of leukemia in adults. The course of the disease is characteristically triphasic: a chronic phase lasting three to six years is followed by transformation to accelerated and then blast phases of short duration. The cause of CML is the translocation of regions of the BCR and ABL genes to form a BCR-ABL fusion gene. In at least 90 percent of cases, this event is a reciprocal translocation termed t(9;22), which forms the Philadelphia (Ph) chromosome. The product of the BCR-ABL gene, the BCR-ABL protein, is a constitutively active protein tyrosine kinase with an important role in the regulation of cell growth.

Thus this fusion product has been found to result in a cancerous growth of the immune system. ABL2 is a product which is a tyrosine kinase resident in the cytoplasm.

Considerable work has been done on ABL and reference is made to that of Wong and Witte as well as O'Hare. Also there is the recent work of Sirvent et al examining Abl in normal and cancer cells.

In the work by O'Hare et al the authors note:

The BCR-ABL signaling network and ABL kinase inhibition.

A, BCR-ABL signaling pathways activated in CML. Dimerization of BCR-ABL triggers autophosphorylation events that activate the kinase and generate docking sites for intermediary adapter proteins such as GRB2. BCR-ABL–dependent signaling facilitates activation of multiple downstream pathways that enforce enhanced survival, inhibition of apoptosis, and perturbation of cell adhesion and migration.

⁹⁴ [TIMP-1 overexpression promotes tumorigenesis of MDA-MB-231 breast cancer cells and alters expression of a subset of cancer promoting genes in vivo distinct from those observed in vitro](http://www.springerlink.com/content/a61k120124411672/), Rebecca L. H. Bigelow, Briana J. Williams, Jennifer L. Carroll, Lisa K. Daves and James A. Cardelli, *Breast Cancer Research and Treatment* Volume 117, Number 1 (2009), 31-44, DOI: 10.1007/s10549-008-0170-7. <http://www.springerlink.com/content/a61k120124411672/>

A subset of these pathways and their constituent transcription factors, serine/threonine-specific kinases, and apoptosis related proteins are shown. A few pathways that were more recently implicated in CML stem cell maintenance and BCR-ABL–mediated disease transformation are shown.

Of note, this is a simplified diagram and many more associations between BCR-ABL and signaling proteins have been reported. BCR-ABL is unstable upon disruption of primary CML cells; therefore, pharmacodynamic evaluation of BCR-ABL activity is performed by monitoring the tyrosine phosphorylation status of either CRKL or STAT5, with CRKL phosphorylation considered the most specific readout.

B, Predicted effectiveness of ABL kinase inhibitors in three therapeutic scenarios: to inhibit native BCR-ABL, to inhibit mutated BCR-ABL, and as a component in the control of CML involving a BCR-ABL–independent alternate lesion⁹⁵.

Now ABL by itself has certain control mechanisms. They are well known and reviewed extensively, refer to Wong and Witte.

10.5 SEMA4D

SEMA4D is also known as CD100. The CD or cluster of determination molecules often are receptors and frequently found on immune system sourced cells. CD100 specifically is characterized as one of Mono migration; with T and B activation; T cell-B cell and T cell-DC interaction. Thus SEMA4D is another immune cell related marker and not one of internal pathway control.

From the work of Gelfand et al we have:⁹⁶

(a) Sema4D signaling in the nervous system. Proteins in the R-Ras pathway are shown in red: in the presence of Sema4D, Rnd1 is recruited to Plexin-B1. Plexin-B1 R-RasGAP activity is activated and downregulates the active form of R-Ras. The decrease of active R-Ras inhibits PI3K–Akt activity, decreasing GSK3 β phosphorylation and, thus, activating it. GSK3 β then phosphorylates and deactivates CRMP2 and causes microtubule disassembly. Proteins in the RhoA pathway are shown in blue: in the presence of Sema4D, receptor tyrosine kinase ErbB2 binds and subsequently phosphorylates Plexin-B1 (as indicated by the double-headed arrow) and then activates PDZ–RhoGEF and LARG, which associate with Plexin-B1. PDZ–RhoGEF and LARG activate RhoA, causing actin depolymerization through ROCK. Proteins in the Rac1 pathway are shown in green. Upon Sema4D binding, activated Plexin-B1 competes for active Rac1 with PAK. The shift in the equilibrium between Plexin-B1- and PAK-bound Rac1 results in decrease of PAK activity, LIMK activity and Cofilin phosphorylation, thus, causing actin depolymerization. So far, this pathway has only been shown in heterologous cells, as indicated

⁹⁵ <http://clincancerres.aacrjournals.org/content/17/2/212.full.pdf+html>

⁹⁶ <http://www.sciencedirect.com/science/article/pii/S096289240900018X>

by the dashed box. Both the actin depolymerization and microtubule disassembly lead to axon growth-cone collapse.

(b) *Sema4D* signaling in the vascular system. Proteins in the *RhoA* pathway are shown in blue: in the presence of *Sema4D*, the receptor tyrosine kinase *Met* binds and phosphorylates *Plexin-B1* (as indicated by the double-headed arrow) and then activates *PDZ-RhoGEF* and *LARG*, which activates *RhoA* and leads to endothelial cell migration through the *ROCK*, *Pyk2* and *PI3K* pathway. It is not clear how this pathway affects actin dynamics or microtubule dynamics in vascular system.

(c) *Sema3A* signaling in the nervous system. *Rac1*-regulating proteins are shown in green: in the presence of *Sema3A*, *FARP2* is released from *Plexin-A1* and activates *Rac1*. *Rac1* then activates *PAK* and *LIMK* and, as a result, phosphorylates *Cofilin*, which finally causes actin depolymerization. *R-Ras*-regulating proteins are shown in red: in the presence of *Sema3A*, *Rac1* facilitates *Rnd1* recruitment to *Plexin-A1*, which induces the *R-RasGAP* activity of *Plexin-A1* and downregulates active *R-Ras*. A decrease in active *R-Ras* downregulates *PI3K-AKT* activity and leads to axon growth-cone collapse through three different pathways: reduced phosphorylation of *GSK3 β* , reduced phosphorylation of *ERM* and activation of *myosin II*. Kinases are shown in blue: in the presence of *Sema3A*, *FARP2* inhibits *PIPK1 γ 661* and suppresses integrin-induced adhesion. *Fer* and *Fes* are activated upon *Sema3A* binding to *Plexin-A1* and phosphorylate and inactivate *CRMP2*, which leads to microtubule disassembly. *Fyn* is also activated after its binding to *Plexin-A1* and inactivates *CRMP2* by phosphorylating and activating *Cdk5*. Both actin depolymerization and microtubule disassembly lead to axon growth-cone collapse.

(d) *Sema3A* signaling in the vascular system. *Sema3A*, through an unknown mechanism (possibly through *Npn-1* and/or a co-receptor, shown as a dashed line and '?'), inhibits *VEGF*-induced activation of *Src* and *FAK* and contributes to angiogenesis. *Sema3A* might also function through *Npn-1* to inhibit integrin-mediated adhesion of endothelial cells to the ECM. *Sema3A* can induce *VE-cadherin* phosphorylation and causes vascular permeability through unknown mechanisms (indicated by '?'), in which *PI3K-Akt* is involved.

In the work of Neufeld and Kessler we have:

The main signal transduction pathways by which *SEMA3A* and *SEMA4D* activate *plexin A1* (*PLEXA1*) or *PLEXB1*...⁹⁷. The information is derived mainly from the study of neuronal cells. The activation of *PLEXA1* by *SEMA3A* (left side) or *PLEXB1* by *SEMA4D* (right side) induces activation and sequestration of *RAC1* and *RND1* by the plexins.

Sequestration of *RAC1* results in reduced phosphorylation of p21-activated kinase 1 (*PAK*), inhibition of LIM domain kinase 1 (*LIMK1*) activity and activation of *cofilin*, which causes actin depolymerization.

⁹⁷ http://www.nature.com/nrc/journal/v8/n8/fig_tab/nrc2404_F5.html

Activation of PLEXA1 by SEMA3A also results in the activation of the tyrosine kinases FYN, FES and FER, which is followed by the recruitment and activation of cyclin-dependent kinase 5 (CDK5), which in turn inactivates collapsin response mediator proteins (CRMPs) such as CRMP2. CRMPs affect microtubule dynamics and the organization of the actin cytoskeleton.

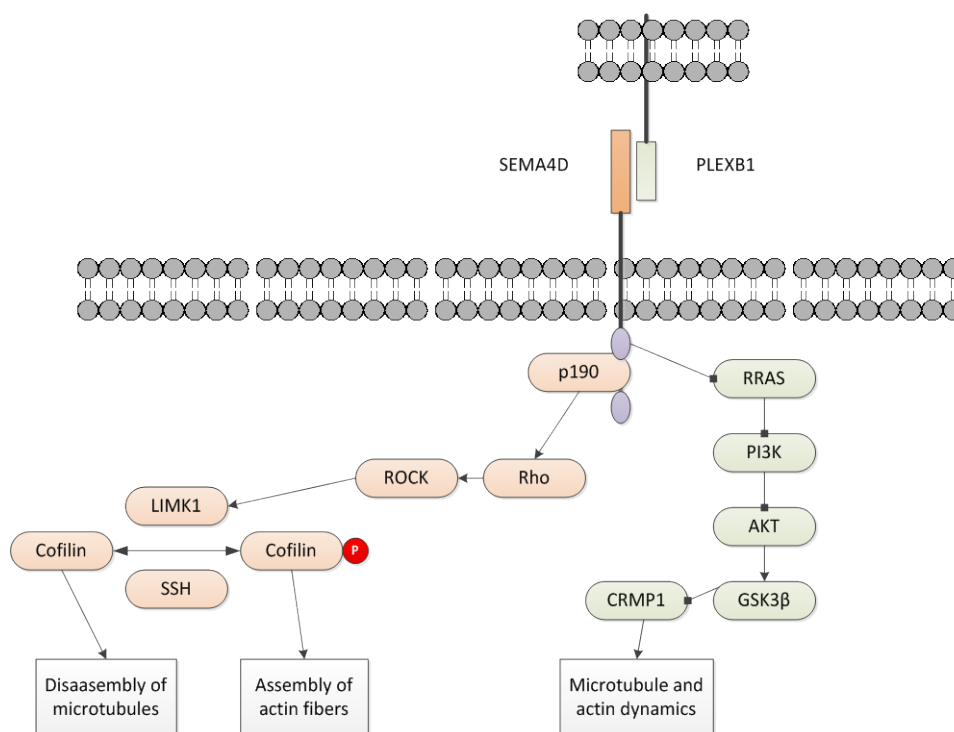
The activation of PLEXA1 also leads to the activation of MICALs (molecules interacting with CasL), which form complexes with CRMPs and are also essential for the effects of SEMA3A on the cytoskeleton.

In the case of SEMA4D, activation of PLEXB1 can also lead to the inactivation of CRMPs through inhibition of phosphoinositide 3-kinase (PI3K) and AKT activation that leads to GSK3 β activation and as a result to the inactivation of CRMPs. The activation of PLEXA1 and PLEXB1 by their respective semaphorins also activates the p190^{RHOGAP} enzyme, which inactivates RHOA and thus contributes to the activation of cofilin by Rho-associated coiled-coil-containing kinase (ROCK) and LIMK1, thereby promoting cofilin activation and actin fibre disassembly.

In contrast with PLEXA1, activated PLEXB1 can also induce the activation of the guanyl nucleotide exchange factors (GEFs) LARG and RHOGEF, thereby counteracting the activity of p190^{RHOGAP}, promoting activation of RHOA and ROCK and leading to increased actin polymerization.

In addition to these short-term effects there are also long-term effects. In the case of SEMA3A, activation of PLEXA1 induces apoptosis of neuronal and endothelial cells, which is manifested by inhibition of extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2) phosphorylation and activation of caspase 3 (indicated in purple). The insert shows the effects of SEMA3A on the actin cytoskeleton of endothelial cells.

We depict below a modified version of their pathway description.



Also below we have from the work of Siderovski and Willard the following discussion of pathway involvement⁹⁸:

Membrane targeting strategies employed by multi-domain RGS proteins.

(A) The R7 RGS proteins form obligate heterodimers with Gβ5 via a Gγ-like sequence (the “GGL” domain) N-terminal to the RGS-box. This GGL/Gβ5 interaction could allow R7 RGS proteins to act as conventional Gβγ subunits in coupling Gα subunits to 7TM receptors, thereby localizing RGS-box-mediated GAP activity to particular receptors. The DEP domain of RGS9-1 interacts with a membrane-anchoring protein (R9AP) analogous interactors may exist for the DEP domains of other R7 subfamily members.

(B) The PDZ domain of RGS12 is able to bind the C-terminus of the IL-8 receptor CXCR2 (at least in vitro). The RGS12 PTB domain binds the synprint (“synaptic protein interaction”) region of the N-type calcium channel (Ca_v2.2); this interaction is dependent on neurotransmitter-mediated phosphorylation of the channel by Src.

(C) The AtRGS1 protein of *Arabidopsis thaliana* (thale cress) has a unique structure for an RGS protein: an N-terminus resembling a 7TM receptor and a C-terminal RGS-box. Although a ligand is not known for the 7TM portion of AtRGS1, a simple sugar is most likely.

(D) The transmembrane receptor Plexin-B1 couples binding of the membrane-bound semaphorin Sema4D to RhoA activation via an interaction with the PDZ domain of PDZ-RhoGEF (and of the related RGS-RhoGEF LARG). Domain abbreviations: IPT, immunoglobulin-like fold found in

⁹⁸ <http://www.biolsci.org/v01p0051.pdf>

plexins, Met and Ron tyrosine kinase receptors, and intracellular transcription factors; PSI, domain found in plexins, semaphorins, and integrins; Sema, semaphorin domain.

The pathway involvement is similar to what we have depicted above.

10.6 ITGAL

ITGAL is integrin alpha L and is also known as CD111, another CD protein and thus another immune response marker and not a pathway marker.

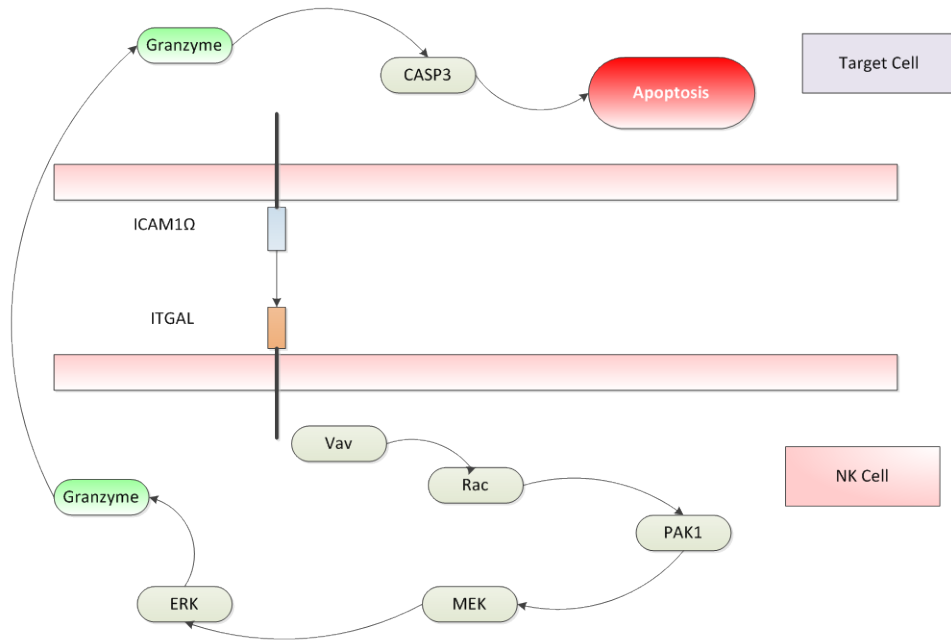
From the KEGG database we have the following additional information⁹⁹:

| | | |
|-------------------|---|---|
| Gene name | ITGAL, CD11A, LFA-1, LFA1A | |
| Definition | integrin, alpha L (antigen CD11A (p180), lymphocyte function-associated antigen 1; alpha polypeptide) | |
| Orthology | K05718 | integrin alpha L |
| Organism | hsa Homo sapiens (human) | |
| Pathway | hsa04514 | Cell adhesion molecules (CAMs) |
| | hsa04650 | Natural killer cell mediated cytotoxicity |
| | hsa04670 | Leukocyte transendothelial migration |
| | hsa04810 | Regulation of actin cytoskeleton |
| | hsa05144 | Malaria |
| | hsa05150 | Staphylococcus aureus infection |
| | hsa05166 | HTLV-I infection |
| | hsa05169 | Epstein-Barr virus infection |
| | hsa05323 | Rheumatoid arthritis |
| | hsa05416 | Viral myocarditis |

From KEGG we have the following pathway¹⁰⁰:

⁹⁹ http://www.genome.jp/dbget-bin/www_bget?hsa:3683

¹⁰⁰ http://www.genome.jp/kegg-bin/show_pathway?hsa04650+3683



Note the connection between the target cell and the NK or Natural Killer cell from the immune system. ITGAL facilitates the apoptosis of the cell. If ITGAL is defective then we have a loss of natural apoptosis.

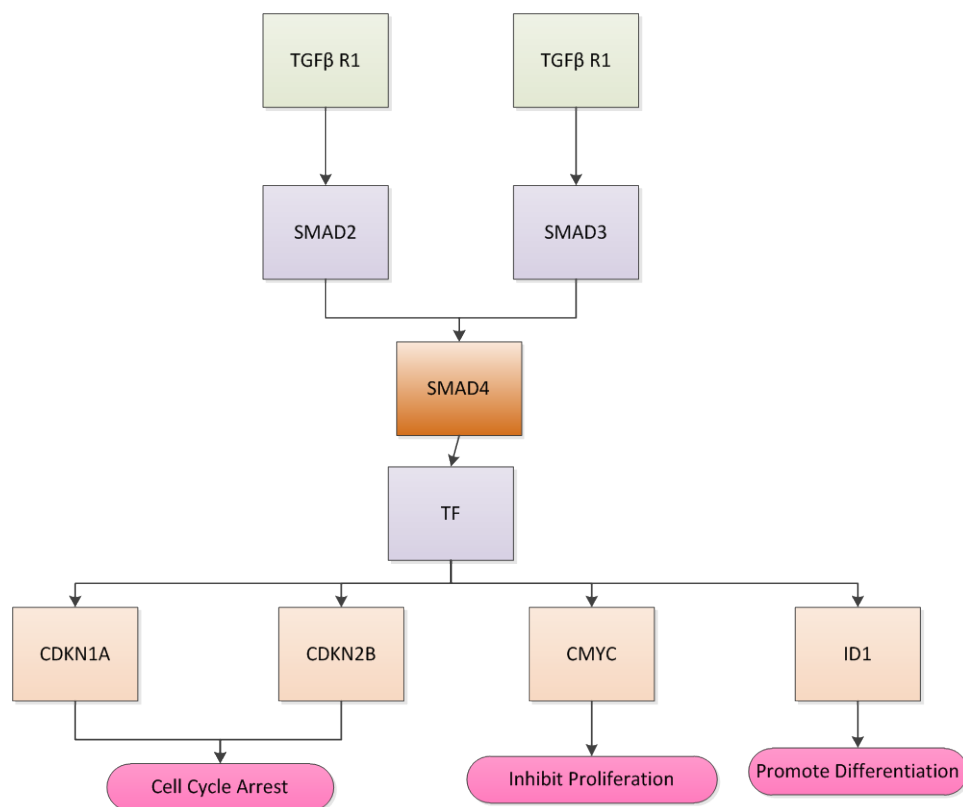
This then is another step in the immune system failing to manage the cell status.

10.7 CDKN1A

CDKN1A is controlled by 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.

Kibel et al have recently examined CDKN1A and CDKN1B specifically in prostate cancers with extensive insight.

We show some of the TGF SMAD signalling below along with its control over the CDKN1A element. We will elaborate this later. Note here that CDKN1A controls apoptosis as well.



SMAD4 controls the G1 to S transition. As stated in NCBI¹⁰¹:

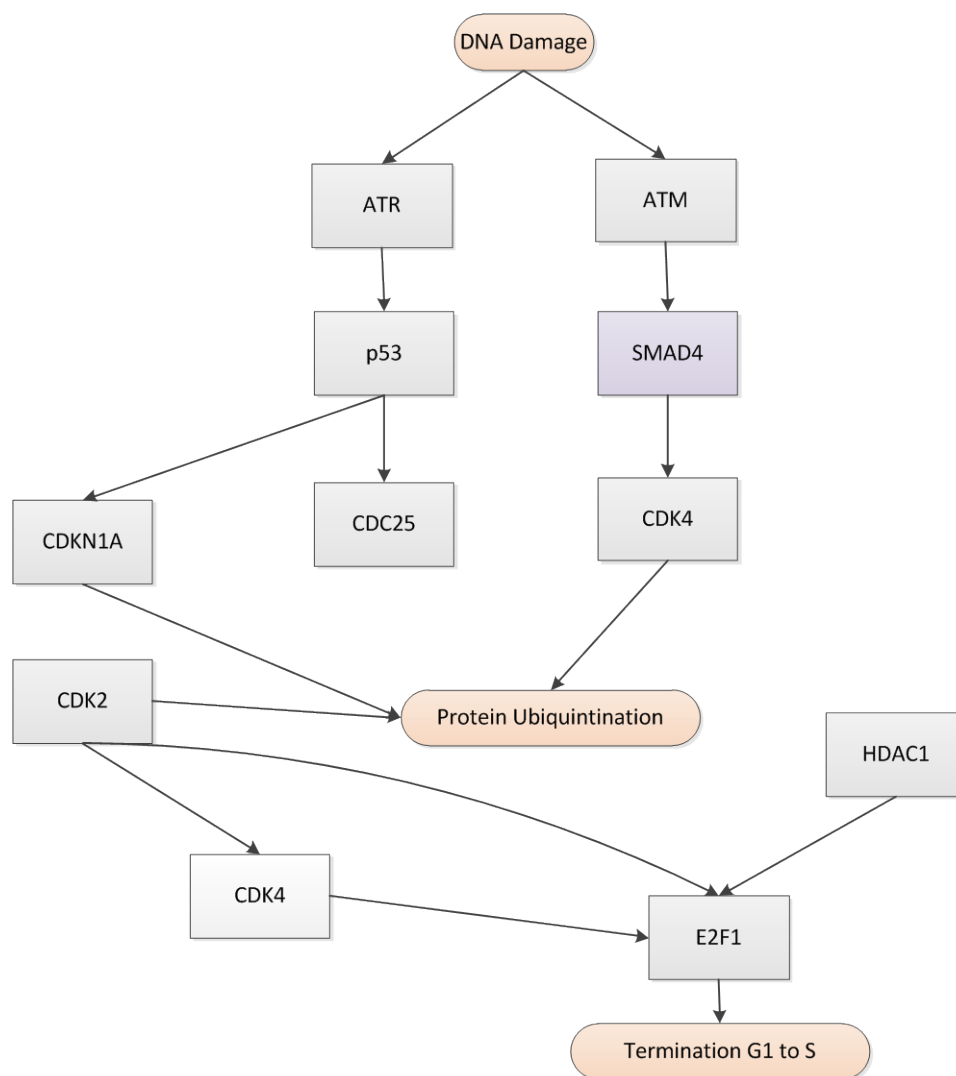
This gene encodes a member of the Smad family of signal transduction proteins. Smad proteins are phosphorylated and activated by transmembrane serine-threonine receptor kinases in response to TGF-beta signaling. The product of this gene forms homomeric complexes and heteromeric complexes with other activated 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 pathway¹⁰²:

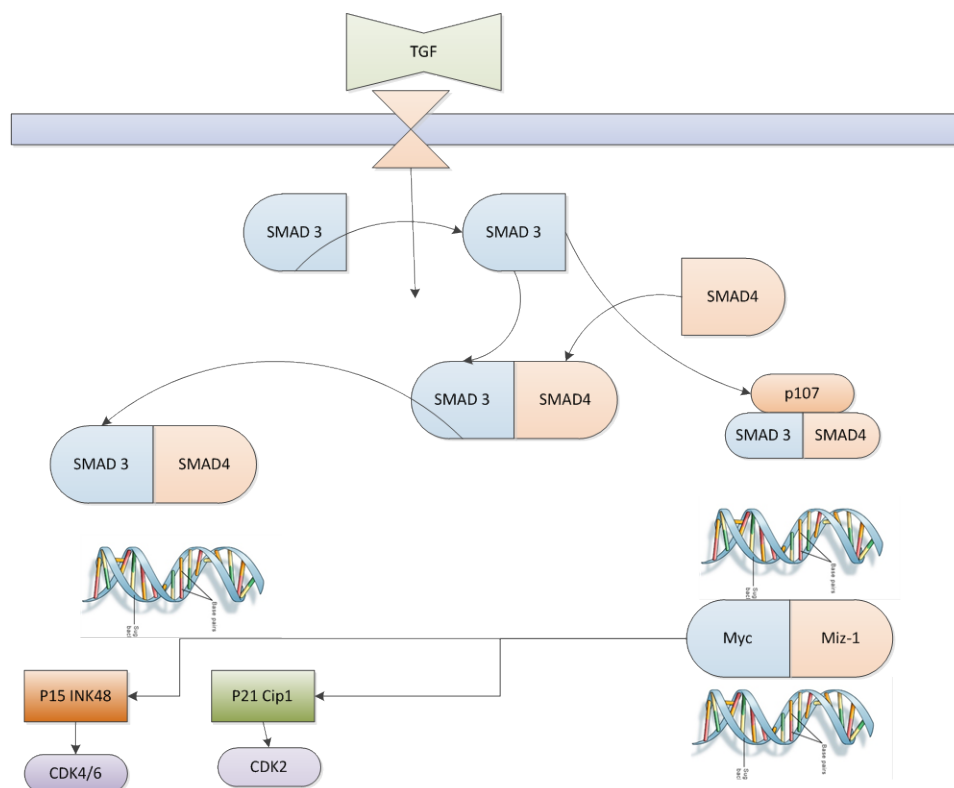
¹⁰¹ <http://www.ncbi.nlm.nih.gov/gene/4089>

¹⁰² 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.

10.8 C1QA

As NCBI states¹⁰³:

This gene encodes a major constituent of the human complement subcomponent C1q. C1q associates with C1r and C1s in order to yield the first component of the serum complement system. Deficiency of C1q has been associated with lupus erythematosus and glomerulonephritis. C1q is composed of 18 polypeptide chains: six A-chains, six B-chains, and six C-chains. Each chain contains a collagen-like region located near the N terminus and a C-terminal globular region. The A-, B-, and C-chains are arranged in the order A-C-B on chromosome 1. This gene encodes the A-chain polypeptide of human complement subcomponent C1q.

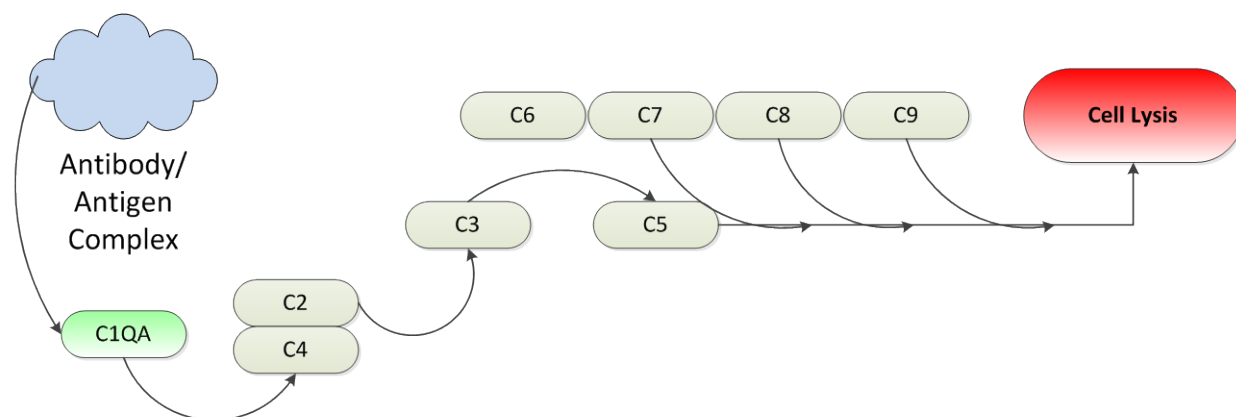
¹⁰³ <http://www.ncbi.nlm.nih.gov/gene/712>

Azzato et al have examined C1QA in breast cancer and they discuss it broadly based presence. They state:

Complement is involved in the primary defence against intravascular microorganisms and has been reported to be involved in the clearance of tumour.... Recently, we have reported an association between expression of C1QA and prognosis in oestrogen receptor (ER)-negative breast cancer... in more than one cohort. We found that ER-negative tumours with overexpression of gene C1QA were associated with a better prognosis. The C1QA gene, located on chromosome 1p36.12, encodes for one of the components of the C1q complex. There are seven single nucleotide polymorphisms (SNPs) catalogued for C1QA on the NCBI database, of which there is only one common SNP (minor allele frequency 45%) located in an exon rs172378 is a synonymous SNP characterised by a G for A substitution at position 361 (A361G).

Thus we have another element from the immune system. It is part of the complement system, not the adaptive part and thus has primitive roots.

Now we depict a selection of its pathway as below (modified from KEGG)¹⁰⁴:



Note that the expression of C1QA is controlling the chain of complement factors which result in cell destruction. Suppression of C1QA then results in loss of this function. C1QA is thus just another factor in the overall control of cell proliferation.

10.9 OBSERVATIONS

There is a seemingly endless progression of genes identified as related to various cancers. All too often they are just noted as almost an incidental finding and as we have discussed before they are often putatively posed with no detailed pathway implications cited.

In this case we see a preponderance of immune system genes expressed albeit in a late stage of cancer. As indicated it is expected that all of these patients are terminal and that we are arguing

¹⁰⁴ http://www.genome.jp/kegg-bin/show_pathway?hsa04610+712

of how soon. The range is from 10 to 40 months. Survival is not an end point; we seem to be arguing over when death occurs. As we had indicated above although it has some prognostic capability it has de minimis quality of care capacity. Thus one wonders why even attempt it other than having some scientific value.

On the other hand we can always view this in a Rosenberg manner and see the immune system kicking in in all manners and fashions. Its failure may then result in metastatic results and rapid death. An interesting question for treatment would be if one could re-stimulate or activate these broken elements and see if they can restore a protective barrier against metastatic results. Rosenberg sought this path in his years of melanoma research. Perhaps this is a means to rejuvenate that to but a later stage of the cancer. Namely we are seeing multiple immune elements failing so what can we achieve to mediate that result.

The problem seen in analyses of this type is that the press all too often exploits its ramifications. This is quite unfortunate for the patients in that they may somehow infer that this discovery may add hope to their plight when in reality it does nothing more than better estimate their demise.

For example there is a quote which states¹⁰⁵:

"There is an urgent need for predictive models that help assess how aggressive the disease is in prostate cancer patients, as survival can vary greatly," said lead investigator William K. Oh, MD, Chief of the Division of Hematology and Medical Oncology of The Tisch Cancer Institute at The Mount Sinai Medical Center. "Our six-gene model, delivered in a simple blood test, will allow clinicians to better determine the course of action for their patients, determine clinical trial eligibility, and lead to more targeted studies in late-stage disease."

This set of tests is not what is desired. We are really desirable of tests which can predict the aggressive nature when the Gleason score is at 6 or less, namely when do we allow, with some sense of safety, for watchful waiting. This report is only for ultimately terminal patients, not those who could survive. This is a classic problem when results like this hit the media, even the professional media. In fact the reports get more exaggerated when we see the results in the popular media.

In summary we may pose the following:

1. There are many of these markers which are immune system related. Is this a common cancer response in the late stages, as much of the literature suggests. If so is the immune system attempting to isolate and defend the body.
2. How does this progress. Somehow one sees snapshots, namely patient A has such and such a profile and we then know when they reach that point the prognosis is bad or very bad. But what are the details of the evolution, do they all follow the same trajectory and if not why not and if so why and what does that mean.

¹⁰⁵ <http://www.onclive.com/web-exclusives/Blood-Test-May-Stratify-Risk-in-Prostate-Cancer>

3. Is there an interaction between the pathways and the immune system or is this just a normal, in the case of cancers, immune response. How much of this is prostate specific and how much is common across a wide variety of malignancies.
4. What does this tell us about potential treatment paths? Can we activate the immune system, can we target it, and is the complement system of special interest. Is this a call to further focus on immune system alternatives?

11 OTHER PROSTATE CANCER ISSUES

We now examine several more recent PCa issues. As will be soon discerned, although as we try to show a logical and linear evolution of understanding PCa and its genetic elements, there is all too often the introduction of new and evolving ones. In this Chapter we show that some of these elements may fit simply while others introduce new and innovative understandings.

11.1 PCA3 AND PROSTATE CANCER

The FDA has recently approved a PCA3 test assay which is owned by a Canadian company, Gen-Probe. This opens up a whole new avenue for examining PCa amongst men. I examine some of the issue here at a fairly high level.

There has been a great deal of discussion regarding PSA and its lack of sufficient specificity and sensitivity to PCa and there is some evidence that PCA3 will improve the situation. This is yet to be determined in extensive clinical trials. One of the problems with PSA is that it is reflective of total prostate volume and it also naturally increases with age. Thus a male of say 70 years of age and with a 70 cc prostate may easily have a PSA of 2.5 just based upon the size and age factors. Likewise if the male were 40 and had a 35 cc prostate then this may be indicative of PCa. In a recent paper by McGarty, we detailed the issue of PSA sampling and the percent change, ie velocity, as a means to assess the nature of the underlying cause. Namely the more prostate basal cells and luminal cells the higher the PSA. As we shall see there is better correlation with PCA3 but the underlying molecular and cellular dynamics do not appear as well defined at this time, namely we have a marker with no clear underlying genomics cause.

The PCA3 measurement is define as follows:

$$PCA3\ Score=1000\left[\frac{[mRNA\ PCA3]}{[mRNA\ PSA]}\right]$$

Where [mRNA PCA3] is the concentration of mRNA of PCA3 and the same for the denominator. The range is such that a PCA3 score of less than 5 gives a very low likelihood of PCa and >35 gives a very high probability. The issue here often is repeat biopsy. The suggestion then is that one use PCA3 as a test for repeat biopsy indication (see Gen-Probe PCA3 documentation). Details on ROC for PCA3 are not broadly available and repeatable at this time.

PCA3 was first discussed in 1999 in a paper by Bussemakers et al, at which time it was called DD3. In their abstract the authors stated at the time:

The DD3 gene was mapped to chromosome 9q21–22, and no homology of DD3 to any gene present in the computer databases was found. Our data indicate that DD3 is one of the most prostate cancer-specific genes yet described, and this makes DD3 a promising marker for the early diagnosis of prostate cancer and provides a powerful tool for the development of new treatment strategies for prostate cancer patients.

It further turns out that PCA3 is a noncoding mRNA and thus there is no protein resultant. This was speculated by Bussemakers et al when they published their work in 1999. The key question seems to be why PCA3 increases when there is a PCa and what the details of the mechanism are. Furthermore where does PCA3 fit within the context of the many pathways we know exist in PCa development?

As Cao and Yao report:

The DD3PCA3 encoding gene is located on chromosome 9 (9q2122). The gene includes four exons and three introns. In PCa, the most frequent mutation is the selective splicing of the second exon. At present, there is a vast body of ongoing studies on PCA3. Hopefully they can further confirm the role of PCA3 in the occurrence and the development of PCa and provide new treatment targets for patients with PCa.

Hessels suggested that using quantitative reverse transcriptase polymerase chain reaction (RT-PCR) for the detection of urine DD3PCA3 was a valuable molecular detection method in patients with PCa and could help reduce unnecessary biopsies. In a multicenter study designed to examine the diagnostic capacity of urine PCA3 detection, the AUC of urine PCA3 detection was 0.66, while the AUC of serum PCA3 detection was merely 0.57. The sensitivity and specificity of PCA3 detection were 65% and 66%, respectively.

Recently, researchers have suggested that serum PSA level plus PCA3 detection was the most promising diagnostic method for PCa. All these studies show that PCA3 is probably an important urine marker for PCa. It also provides a new clue for developing noninvasive detection methods for PCa. Hence, PCA3 may have considerable significance in multiple tumor marker screening of patients for PCa in the future.

Thus one of the questions is what is PCA3 and why does it reflect PCa presence. We know that we are measuring mRNA concentrations, and we know that in measuring them we have experimental evidence that PSA reflects total cell concentration. But what of PCA3, what does that reflect.

In a recent paper by Clarke et al the authors attempt to clarify what the role of PCA3 is.

In order to understand further the importance of the PCA3 gene in PCa we undertook a more detailed investigation of this gene and its chromosomal locus. This investigation points to a considerably more complex transcriptional unit for PCA3 than originally reported including additional novel exons. We describe a number of novel PCA3 splice variants with more specific expression in PCa tissues and metastases.

We also demonstrate that PCA3 is embedded in the intron of a second gene, BMCC1, a gene implicated in controlling oncogenic transformation and that both genes showed increased expression in PCa and metastases. The absence of a TATA box element within a human gene promoter has been associated with promiscuous transcriptional initiation. The PCA3 gene does not contain an upstream TATA sequence and it was therefore of interest to determine whether any additional transcription initiation sites existed for PCA3

Perhaps this relationship to BMCC1 may lead to some insight. They continue:

BMCC1 is upregulated in PCa and androgen inducible Since PCA3 is upregulated in PCa and since we showed here that this gene is embedded in a second gene BMCC1, implicated in cellular proliferation, we determined whether BMCC1 was also differentially regulated in PCa. We used a set of RT-PCR primers that span that region of the BMCC1 gene (exons 6 and 7), specific for the full-length BMCC1-1 transcript. Expression of BMCC1-1 was evident in normal prostate and BPH specimens and was upregulated in PCa and metastases (Fig 4A, Supplementary Fig. S4).

This was confirmed using primers corresponding to the BCH C-terminal region of BMCC1 and for BMCC1-2. Indeed amplification of this isoform gave better discrimination between PCa and BPH (Fig. 4A, upper panel). Extending these experiments to PCa and other cell lines revealed that both genes were highly expressed, specifically in the PCa cell line LNCaP. In addition BMCC1-1 was detected in a second PCa cell line DU145 but at lower levels. PCA3 is also expressed in DU145 but required further rounds of amplification for detection. The shorter BMCC1 isoforms (BMCC1-3 and/or BMCC1-4) were also detected (using primers specific for the BCH region) in an EBV-transformed lymphoblastoid cell line (JHP), but the longer BMCC1-1 isoform was not detected.

Previous data have shown that the level of PCA3 can be induced in LNCaP cells after treatment with dihydrotestosterone, which mimics the effects of binding of the androgen receptor (DHT). We determined whether BMCC1-1 was also responsive to hormonal induction. The results Demonstrate that both PCA3 and BMCC1 are maximally induced in the LNCaP cell line at a concentration of 0.5 mM DHT.

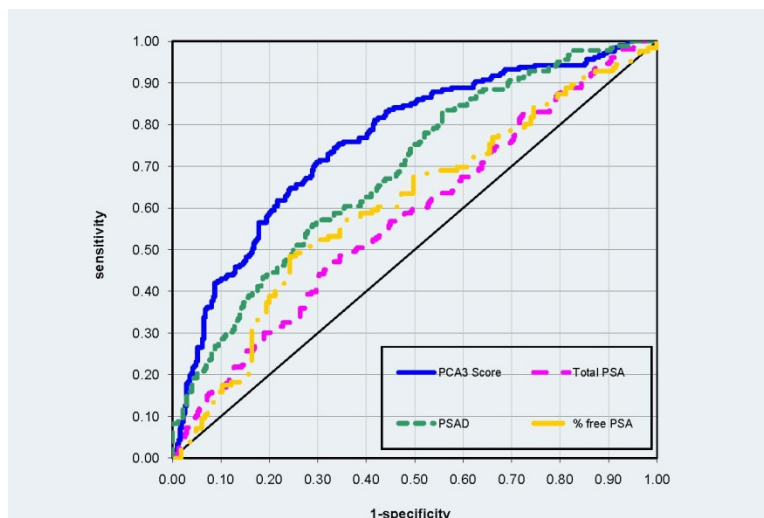
Thus there seems to be some means of related induction but again no definitive relationship to well defined pathways.

The following is the PCA3 and PSA ROC for comparison. Note the following (see de la Taille):

The area under the curve of the receiver operating characteristics (AUC ROC) of the PCA3 Score was compared with that of serum total PSA, PSAD and %free PSA. The diagnostic accuracy of the PCA3 Score was statistically significantly better than that of serum total PSA, PSAD and %free PSA.

The greatest diagnostic accuracy of the PCA3 Score was obtained at a cut-off of 35: specificity 76% and sensitivity 64%. At a sensitivity of 80%, the PCA3 Score specificity of 58% was higher than the 44% for PSAD and 27% for serum total PSA and %free PSA.

The following from de la Taille is the comparative ROC. It appears that from the limited data available that the ROC curve is substantially better with PCA3 than PSA alone.



The key questions remaining are:

1. What pathway elements does PCA3 reflect? What genetically is happening and what is the underlying system model. This is always an issue. As with primary medicine we have underlying physiology, here we must have some underlying genomics.
2. What are the cellular mechanisms which control PCA3? Again this is a pathways issue.
3. How sustainable is PCA3 ROC for this assay. Many tests have been done and FDA approval is merely acceptance of some limited tests.
4. How does one relate PSA and PCA3? Note that the PCA3 measure does reflect PSA concentration, so we have not abandoned PSA.
5. Why do we normalize PCA3 on PSA? If PSA has such a variability are we normalizing on something which is inherently unpredictable?

11.2 HOMEBOX

The Homeobox and its related genes have played an interesting but challenging role in developmental biology and now in cancer pathways. The genes related to this 180 base pair section of DNA are the genes which control the development of organs and the time at which these development occur. Furthermore the structure of this gene collection is preserved across a dramatically large number of species, the human included. Thus it was interesting to see a paper in NEJM discussing the mutation of a specific Homeobox gene, HOX B 13, as relates to prostate cancer.

In the recent NEJM paper by Ewing et al the conclusion of the authors is stated as:

*The novel **HOXB13 G84E** variant is associated with a significantly increased risk of hereditary prostate cancer. Although the variant accounts for a small fraction of all prostate cancers, this finding has implications for prostate-cancer risk assessment and may provide new mechanistic insights into this common cancer.*

Now this appears as a significant new finding and we would like to examine this a bit. The HOX genes are quite unique in their functioning. They are built about a core Homeobox segment, which is preserved across chromosomes and species, and is then connected with variable regions on differing chromosomes to generate some 4X13 possible genes (HOX (A,B,C,D) (1...13)). These genes are core to the morphological and embryological development of a broad range of species.

Now HOX B 13 is one of many Homeobox based genes. These genes are distributed across 4 chromosomes and have a fixed part called the homeobox part and a variable part. The gene is created as below:



HOX B 13 for example uses the Homeobox segment of 180 BP and is connected to variable region creating a unique gene but the binding site is based upon the common Homeobox segment. Homeobox is preserved across species.



Homeobox genes are clustered in the chromosomes and are expressed in the body in the same order in which they occur in the chromosomal DNA. The HOX genes, the concatenation of the respective Homeobox and its variable part are named by chromosome location as A, B, C, D, and then by number 1 through 13 at present. The number reflects what makes the Homeobox genes of interest, namely the genes control the development of the embryos, namely they control what cells do as a part of the development of an entity.

The process goes from head to tail, and the numbering goes from the earliest or anterior to the latest or posterior elements in the development process. Thus HOX A 1 relates to an early development and HOX B 13 would refer to a later development of the embryo. The sequencing is shown below.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
|---|-------|---|---|---|---|---|---|---|---|----|----|----|--------|
| A | HOXA1 | | | | | | | | | | | | |
| B | | | | | | | | | | | | | HOXB13 |
| C | | | | | | | | | | | | | |
| D | | | | | | | | | | | | | |

Retinoic acid activates the Homeobox genes sequentially in development.

Now the Ewing study examined patients with specific changes:

Given the consistent evidence of prostate-cancer linkage to 17q21-22 markers in our multiplex families with hereditary prostate cancer, we designed a targeted sequencing strategy to analyze 2009 exons of 202 genes contained in the most likely genomic interval defined by our fine-mapping studies. ... Probands from four families were observed to have the same nonsynonymous mutation in HOXB13, a change of adenosine for guanine (transition, c.251G→A) in the second position of codon 84 (GGA→GAA), resulting in a nonconservative substitution of glutamic acid for glycine (G84E)

The question is perhaps: where does the term Homeobox come from. From Gehring and Hiromi we have the definition:

The term "homeosis" (originally spelled "homoeosis") was proposed by Bateson (8) to describe the transformation of one structure of the body into the homologous structure of another body segment. Homeotic transformation can result, for example, from abnormal regeneration of amputated structures (epigenetically) or from germ-line mutations

Thus the Homeobox genes are key to the development of embryos. They also lead to the discussions

Scott states:

Homeotic genes control cell fates during the development of all animals, as was first revealed by studies of the Drosophila homeotic gene complexes ... Many of these genes contain a homeobox, a 180 bp sequence of DNA which encodes an evolutionarily conserved DNA binding domain, the homeodomain ... A plethora of mammalian homeobox genes have been reported, among which 38 are located in four clusters. A new nomenclature for the mammalian Hox genes, approved ...

The new names take advantage of the elegant arrangement of the genes to provide a logical nomenclature system rather than the names given when the genes were discovered. The new system is initially designed only for vertebrate genes, although it is to be hoped that similar systems will be useful, and adopted, for other animals. In order to preserve as much clarity in the literature as possible, it has been agreed by a large number of workers in the field and by the nomenclature committees that homeobox genes not located within the Hox complexes should not be given names containing the word 'Hox'. There are four clusters of Hox genes ... now to be known as A, B, C, and D. Based on sequence similarity the genes can be sorted into 13 'paralog' groups, each group having, in most cases, a representative in each complex. The order of paralogs along the chromosome is preserved in the four complexes. The genes within a complex are transcribed in the same direction and are numbered according to their paralog group from 1 at the 3' end to 13 at the 5' end. In several cases a representative of a paralog group is absent from a complex, in which case the corresponding gene number is omitted ...

HOX genes are key to the development of the embryo; it creates the head to tail and sets up the control of the development of the organs. As Lohmann and McGinnis report:

Hox genes play a major role in the morphological diversification of the anteroposterior body axis of animal embryos by switching the fates of segments between alternative developmental pathways. In their role of controlling segment diversity, Hox proteins are responsible for many different morphological structures and cell types within a given segment. But it is still largely a mystery how a single Hox gene can determine a morphological trait at a specific location within a segment, and why that trait does not appear elsewhere in the same segment or in other segments.

... morphological and transcriptional responses to Hox genes can be highly local, sometimes only in a single cell, allowing one Hox gene to control a cavalcade of different traits within one segment and between different segments, depending on the information present. Another important lesson that we can learn from the papers of Rozowski and Akam and Brodu et al. is that, during development, Hox genes act at all levels in the developmental hierarchy. If they act very far down in the hierarchy, as in these two cases, then the output is subtle, with Hox genes acting as cell-type switches rather than as major developmental pathway switches. If they are acting (apparently) far up in the hierarchy, then the fate switch is more dramatic, which is most beautifully demonstrated in the famous four-winged fly. But even at this general level, context is still crucial: loss of Ubx in the haltere does not generate a leg, but a wing.

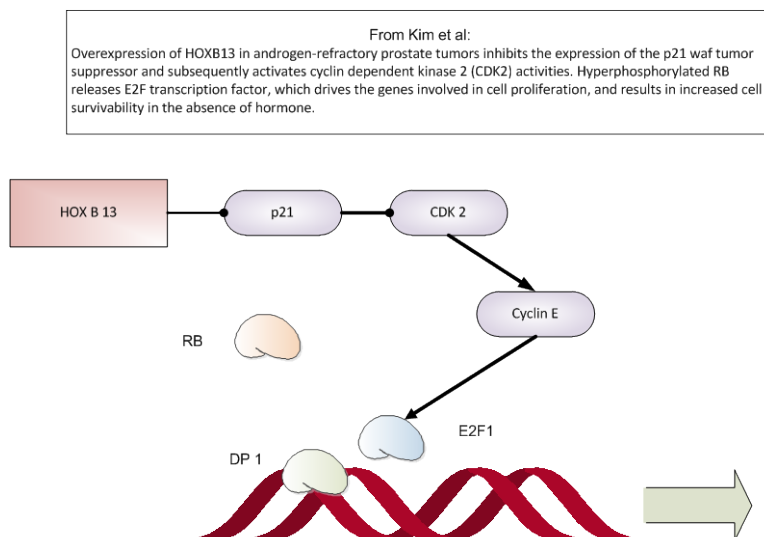
There are many debates still raging regarding Homeobox and Robert presents an interesting report summarizing some of them. His paper is worth the reading. It builds on the evo-devo issue, evolution and development, the ontogeny recapitulates ontogeny. Namely if the same HOX genes are present across many species, and preserved in structure, then is there really an underlying commonality across species.

We provide the details on the various HOX genes below. They all have the form as we had shown earlier and they are all numbered in a sequence consistent with what we have shown earlier.

| Type | Location | Genes Produced |
|-------|---------------|--|
| HOX A | chromosome 7 | HOXA1, HOXA2, HOXA3, HOXA4, HOXA5, HOXA6, HOXA7, HOXA9, HOXA10, HOXA11, HOXA13 |
| HOX B | chromosome 17 | HOXB1, HOXB2, HOXB3, HOXB4, HOXB5, HOXB6, HOXB7, HOXB8, HOXB9, HOXB13 |
| HOX C | chromosome 12 | HOXC4, HOXC5, HOXC6, HOXC8, HOXC9, HOXC10, HOXC11, HOXC12, HOXC13 |
| HOX D | chromosome 2 | HOXD1, HOXD3, HOXD4, HOXD8, HOXD9, HOXD10, HOXD11, HOXD12, HOXD13 |

Note all HOX B are from Chromosome 17. In particular HOX B 13 is 17q21-22 region¹⁰⁶.

We now show from Kim et al the development of the pathway for the HOX B 13 that we have been discussing. It inhibits CDK and that in turn inhibits the activation via E2F of the cell cycle. It is the inhibition of the cell cycle that is of the most concern.



As Kim et al demonstrate the HOX B 13 blocks p21 and in turn CDK2 keeping the RB pathway from entering the cell into cell cycle reproduction. They state:

Taken together, the results of this study demonstrated the presence of a novel pathway that helps understand androgen-independent survival of prostate cancer cells. These findings suggest that upregulation of HOXB13 is associated with an additive growth advantage of prostate cancer cells in the absence of or low androgen concentrations, by the regulation of p21-mediated E2F signaling.

Now Ewing et. al. concludes as follows:

In summary, we have used linkage analysis in combination with targeted massively parallel sequencing to identify a recurrent mutation in HOXB13 that is associated with early-onset and hereditary prostate cancer. From a clinical perspective, testing for germline mutations in BRCA1/2 is recommended in some families, since mutations in these breast-cancer-susceptibility genes are associated with elevations in the risk of prostate cancer, particularly for BRCA2. However, neither of these genes has been shown to contribute to hereditary prostate cancer. HOXB13 G84E is associated with a significantly increased risk of hereditary prostate cancer. This work suggests that future DNA sequencing studies using next-generation technology and study populations enriched for genetic influence (as evidenced by an early age at onset and positive family history) may identify additional rare variants that will contribute to familial clustering of prostate cancer. Although HOXB13 mutations will be identified in a minority of

¹⁰⁶ http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene&cmd=retrieve&dopt=default&rn=1&list_uids=10481

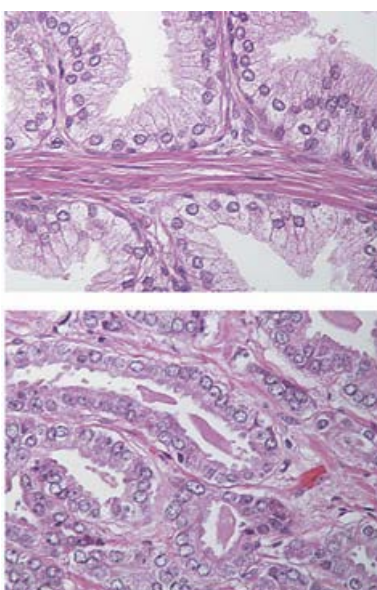
men with prostate cancer, rare genetic lesions can identify pathways that are found to be abnormal in more common, sporadic cases.

This leaves one to somewhat guess as to how prevalent this mutation is. It also begs the question of why as a mutation which is apparently inherited the progression of the cancer is so slow. Ewing et al show that the odds ratio can be as high as 32.5:1 when the mutation is present. The age at diagnosis is lower with an odds ratio of 2:1 but with the problem one sees in pathway control one wonders why the cancer does not appear much earlier as seen in BRCA.

Thus this paper raises several questions:

1. The Homeobox mutation is a predisposing genetic risk factor. If tested and found positive for the factor what should be done next? Mastectomy is often what BRCA patients undergo, does this mean prophylactic prostatectomy?
2. The pathway seems to be somewhat understood. The E2F family controls the pathway and HOX B 13 controls that pathway. It blocks it to some degree. What can happen to HOX B 13 to cause this change in non-mutated individuals?
3. Can the disease propensity be regulated by genetic pathway control, is this possible as an alternative prophylactic measure?
4. What other pathway elements should be considered.
5. Most importantly, why does it take so long for the cancer to develop, are there precursor hits somewhere and this just eliminates other hits?

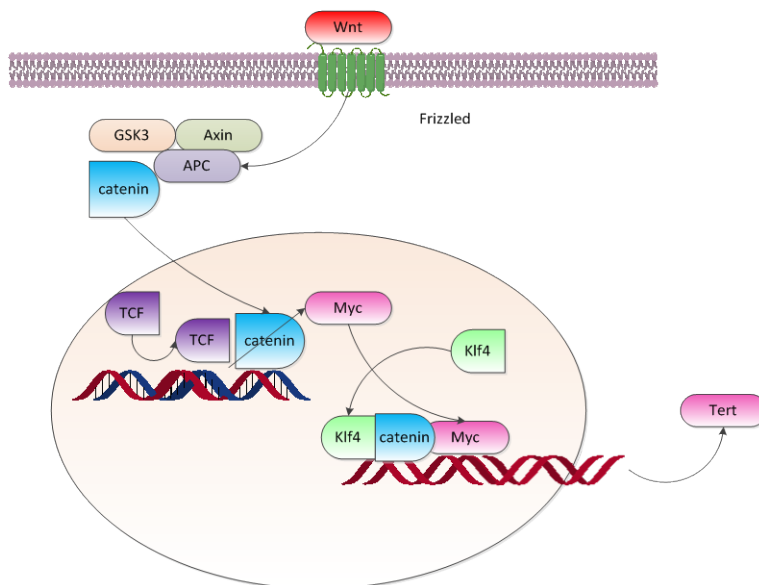
Ewing et al have an interesting slide showing normal versus HOX B 13 prostate cells and we replicate it below from the paper.



In the top slide we see well-structured prostate cells with basal and luminal layers not showing and aberrant growth, no PIN. In the slide below from a HOX B 13 patient with a mutation of the form: GGA to GAA Glycine Glutamic acid (See Ewing et al).

11.3 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 in Science presents some interesting work on Wnt and TERT.¹⁰⁷



Wnt is an extra cellular signaling protein and it attaches to Frizzled a receptor and sets off a cascade that moves β-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:

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

¹⁰⁷ http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene&cmd=retrieve&dopt=default&rn=1&list_uids=10481

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 κ B (NF- κ B), 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.

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.

11.4 BAD CELLS USING GOOD CELLS: METASTASIS

In the Nature article by Straussman et al, they state (also see write up by Carpenter):

Drug resistance presents a challenge to the treatment of cancer patients. Many studies have focused on cell-autonomous mechanisms of drug resistance. By contrast, we proposed that the tumour micro-environment confers innate resistance to therapy.

Carpenter states:

The presence of these cancer-assisting proteins in the stromal tissue that surrounds solid tumours could help to explain why targeted drug therapies rapidly lose their potency.

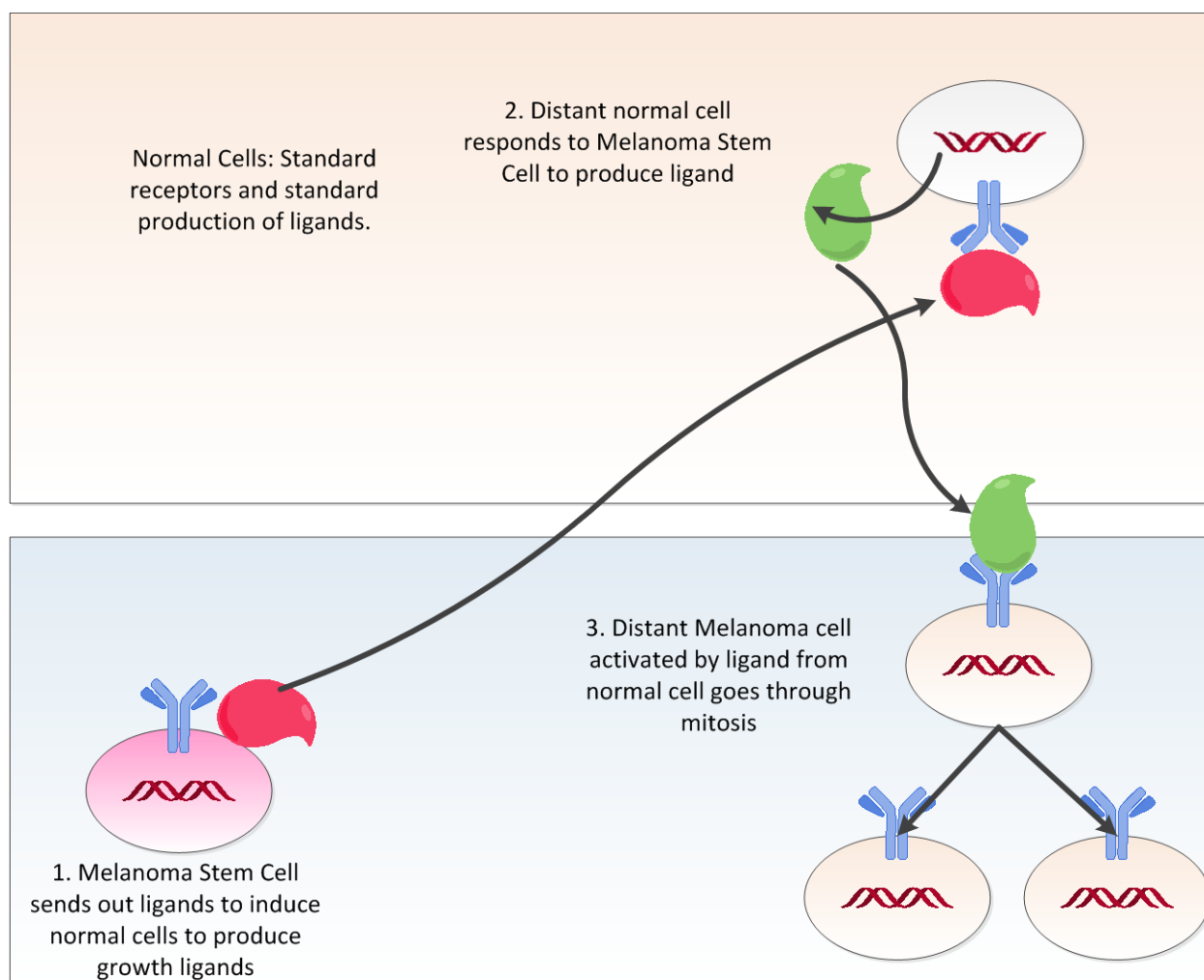
Targeted cancer therapies are a class of drugs tailored to a cancer's genetic make-up. They work by identifying mutations that accelerate the growth of cancer cells and selectively blocking copies of the mutated proteins. Although such treatments avoid the side effects associated with

conventional chemotherapy, their effectiveness tends to be short-lived. For example, patients treated with the recently approved drug vemurafenib initially show dramatic recovery from advanced melanoma, but in most cases the cancer returns within a few months.

The Carpenter article concludes:

One of the most startling results of the teams' experiments was the discovery that a protein called hepatocyte growth factor (HGF) boosts melanoma's resistance to treatment with vemurafenib. Intrigued by this result, both teams looked at blood samples from people who had undergone treatment with vemurafenib, and found the higher a patient's HGF levels, the less likely they were to remain in remission.

We propose an alternative but what we believe to be a consistent interpretation. Consider the example below. We have conjectured based upon modeling that cancer may act as a separate entity on the human host and further that it uses the human host not only for nutrients but for communications. In fact using the results from this paper one can construct a verifiable model of a bi-system distributed environment. Here the melanoma uses a stem cell to communicate at a distance.



The above is a hypothetical example:

1. There exists a melanoma stem cell. It can produce ligands which manage to use the body's distribution system; blood or lymphatic.
2. The ligands use normal health cells which are to be activated and in turn produce at a distant site growth ligands at that site.
3. At the distant site we have Melanoma non stem cells which respond to this massive influx, an amplifier system if you will, to make the non-stem melanoma cells to proliferate.

Just an interesting but possible physical interpretation.

11.5 CANCER CELLS AND THE ENVIRONMENT

There is an interesting piece on Eureka talking about how researchers now believe the environment, micro environment, can be a controller to cancer cell.

They state:

The research team has found that normal cells that reside within the tumor, part of the tumor microenvironment, may supply factors that help cancer cells grow and survive despite the presence of anti-cancer drugs. These findings appear online this week in a paper published in Nature.

"Historically, researchers would go to great lengths to pluck out tumor cells from a sample and discard the rest of the tissue," said senior author Todd Golub director of the Broad's Cancer Program and Charles A. Dana Investigator in Human Cancer Genetics at the Dana-Farber Cancer Institute. Golub is also a professor at Harvard Medical School and an investigator at Howard Hughes Medical Institute. "But what we're finding now is that those non-tumor cells that make up the microenvironment may be an important source of drug resistance."

We have argued likewise in one of our recent White Papers. We argued that such cancers as melanoma have a compelling model for metastasis which uses both short distance micro environment control as well as long distance macro environment signalling.

Namely we have modeled melanoma metastasis as a quasi-distinct organism using the human as a host and specifically using the host extracellular signalling as a means for allowing the stem cell to effect metastasis at a distance.

This laboratory effort is truly worth following.

11.6 STOP AND GO GENES

In a recent paper by Solimini et al the authors discuss the concepts of STOP and GO genes and carcinogenesis¹⁰⁸. 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 .

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.

¹⁰⁸ Solimini, N., et al, Recurrent Hemizygous Deletions in Cancers May Optimize Proliferative Potential, Science, 6 JULY 2012 VOL 337, p 104.

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.

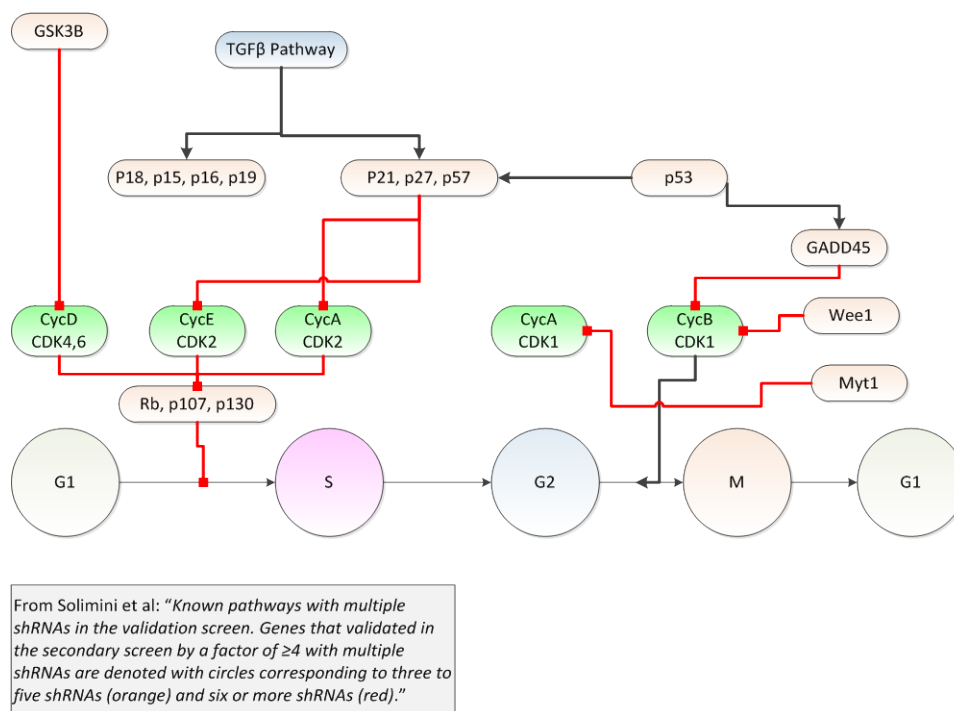
Because hundreds to thousands of genes are hemizygously deleted per tumor, this mechanism may help to drive tumorigenesis across many cancer types.

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 modified, and also not that they have short hairpin RNAs (shRNAs)).



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.

11.7 CNV AND PROSTATE CANCER

Each day we see more relationships between genes, SNPs, miRNA, and now CNVs, copy number variants, to some form of cancer. There is a recent paper in The American Journal of Pathology which relates CNVs to prostate cancer, PCa, and the prognosis of the disease.

We start with a brief discussion of a CNV. It is defined as follows:

Copy number variant (CNV): A duplication or deletion event involving >1 kb of DNA.

Simply a CNV may be the addition of one or more copies of a gene or part thereof in a chromosome. It simply adds to the chromosome. They are quite common and thus are seen frequently. Some are related to certain genetically inherited disorders. In the paper at point they are used to ascertain potentially prognostic data.

From the paper by Yu et al¹⁰⁹:

The prediction of prostate cancer clinical outcome remains a major challenge after the diagnosis, even with improved early detection by prostate-specific antigen (PSA) monitoring.

To evaluate whether copy number variation (CNV) of the genomes in prostate cancer tumor, in benign prostate tissues adjacent to the tumor (AT), and in the blood of patients with prostate cancer predicts biochemical (PSA) relapse and the kinetics of relapse, 241 samples

¹⁰⁹ Yu, Y., et al, Genome Abnormalities Precede Prostate Cancer and Predict Clinical Relapse, The American Journal of Pathology - June 2012 (Vol. 180, Issue 6, Pages 2240-2248, DOI: 10.1016/j.ajpath.2012.03.008). <http://www.journals.elsevierhealth.com/periodicals/ajpa/article/S0002-9440%2812%2900241-6/abstract>

(104 tumor, 49 matched AT, 85 matched blood, and 3 cell lines) were analyzed using Affymetrix SNP 6.0 chips.

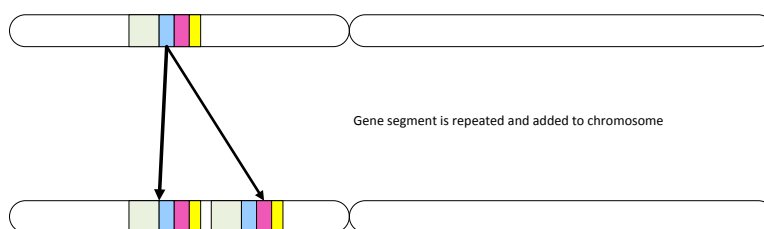
By using gene-specific CNV from tumor, the genome model correctly predicted 73% (receiver operating characteristic $P = 0.003$) cases for relapse and 75% ($P < 0.001$) cases for short PSA doubling time (PSADT, <4 months). The gene-specific CNV model from AT correctly predicted 67% ($P = 0.041$) cases for relapse and 77% ($P = 0.015$) cases for short PSADT. By using median-sized CNV from blood, the genome model correctly predicted 81% ($P < 0.001$) cases for relapse and 69% ($P = 0.001$) cases for short PSADT.

By using median-sized CNV from tumor, the genome model correctly predicted 75% ($P < 0.001$) cases for relapse and 80% ($P < 0.001$) cases for short PSADT. For the first time, our analysis indicates that genomic abnormalities in either benign or malignant tissues are predictive of the clinical outcome of a malignancy.

We briefly examine the CNV in general. In the work of Freeman et al we have¹¹⁰:

DNA copy number variation has long been associated with specific chromosomal rearrangements and genomic disorders, but its ubiquity in mammalian genomes was not fully realized until recently. Although our understanding of the extent of this variation is still developing, it seems likely that, at least in humans, copy number variants (CNVs) account for a substantial amount of genetic variation. Since many CNVs include genes that result in differential levels of gene expression, CNVs may account for a significant proportion of normal phenotypic variation. Current efforts are directed toward a more comprehensive cataloging and characterization of CNVs that will provide the basis for determining how genomic diversity impacts biological function, evolution, and common human diseases.

We show an example of a CNV below graphically.



Here we have depicted a gene, the multicolor object in a chromosome and we have shown a CNV with an identical copy of the gene in the same chromosome. The authors continue:

CNVs often occur in regions reported to contain, or be flanked by, large homologous repeats or segmental duplications. Segmental duplications could arise by tandem repetition of a DNA

¹¹⁰ Freeman, J., Copy number variation: New insights in genome diversity, Published in Advance June 29, 2006, doi: 10.1101/gr.3677206 *Genome Res.* 2006. 16: 949-961
<http://genome.cshlp.org/content/16/8/949.full.html#ref-list-1>

segment followed by subsequent rearrangements that place the duplicated copies at different chromosomal loci. Alternatively, segmental duplications could arise via a duplicative transposition-like process: copying a genomic fragment while transposing it from one location to another

It must be noted that these are identical duplications of the genes, or segments thereof. If of a gene the segment can be transcribed as easily as the original. This raises the question that the resulting translated protein is at a potential multiple level of concentration, although this may not necessarily be the case. They continue:

Large duplications and deletions have been known for some time to be related to the presentation of specific genetic disorders, presumably as a result of copy number changes involving dosage-sensitive developmental genes. This has led to the establishment of genetic diagnostic tests for certain, well-characterized microdeletion and microduplication syndromes (e.g., Angelman syndrome, DiGeorge syndrome, Charcot-Marie-Tooth disease, etc.).

If a de novo chromosomal aberration is recognized in a patient with a constitutional genetic abnormality (i.e., follow-up studies fail to reveal a similar chromosomal aberration in either of the two parents, and non-paternity has been excluded) and the aberration is not one of the dozen or so well-known common chromosomal polymorphisms (e.g., inversion on chromosome 9), the aberration is assumed to be the cause of the clinically recognized abnormal phenotype.

Finally the CNVs are not necessarily related to disorders. Some have CNV but many CNV are not noticeable. They thus state:

CNVs that do not directly result in early onset, highly penetrant genomic disorders may consequently be considered to be neutral in function, but afterward shown to play a role in later onset genomic disorders or common diseases. Analyses of the functional attributes of currently known CNVs reveal a remarkable enrichment for genes that are relevant to molecular–environmental interactions and influence our response to specific environmental stimuli.

These include, but are not limited to, processes involving drug detoxification (e.g., glutathione-S-transferase, cytochrome P450 genes, and carboxylesterase gene families), immune response and inflammation (e.g., leukocyte immunoglobulin-like receptor, defensin, and APOBEC gene families), surface integrity (e.g., late epidermal cornified envelope and mucin gene families), and surface antigens (e.g., galectin, melanoma antigen gene, and rhesus blood group gene families). Likewise, some CNVs encompass genes that may contribute to interindividual variation in drug responses, as well as in immune defense and disease resistance/susceptibility among humans.

From the Thorne and District Gazette¹¹¹:

This study was appropriately designed to see whether patients who have different outcomes have differences in copy number variation. However, before this technique can be used as a test, it will have to be trialled on a much larger cohort of people, so that researchers can get a clearer picture of its use in clinical settings. For example, researchers will need to know how often the

¹¹¹ <http://www.thornegazette.co.uk/news/health/behind-the-headlines/dna-blood-test-for-prostate-cancer-1-4606100>

test might miss patients that are likely to relapse, and also how often the test incorrectly suggests a person's cancer is likely to relapse, which could lead them to have unnecessary further treatment. Also, as the authors note, the techniques used in this study need high-quality DNA, so may be difficult and expensive to perform...

The article then states regarding the outcomes:

- 1. Approximately one-third of the patients had a relapse soon after surgery, with a median time to progression of 1.9 months.*
- 2. One-third had a relapse but much more slowly, with a median time to progression of 47.4 months.*
- 3. One-third of patients in the cohort were free of cancer for at least five years.*

Based on the associations they found, the researchers developed an algorithm for predicting whether a patient would relapse, and how quickly they would relapse. This was based on whether the genetic code at specific locations was repeated or deleted, or on the size of copy number variation found across a person's genome. They then tested their prediction model on an additional 25 samples.

They then conclude:

The researchers found that the prostate cancer samples had a large number of genetic abnormalities. (i) Deletions of specific regions occurred at high frequency, and amplification (abnormal repetitions) of other regions occurred in a subset of samples. (ii) Healthy tissue adjacent to a tumour also had similar amplification and deletion patterns. (iii) The blood of patients with prostate cancer also contained copy number variations, and some of these variations occurred in the same locations within the DNA as they had in the prostate cancer samples.

The researchers then developed a tool to predict whether a cancer would relapse based on DNA regions that had a significant proportion of amplification or deletion in prostate tissue samples from patients who relapsed, but not in patients who did not relapse. The prediction model looking at cancer tissue samples could predict a relapse correctly 73% of the time. (i) It had a 75% accuracy for predicting rapid relapse. (ii) The prediction model based on examining healthy tissue samples could predict a relapse 67% of the time. (iii) It had a 77% accuracy for predicting rapid relapse. (iv) This blood-based prediction model had an accuracy of 81% for predicting relapse, and a 69% accuracy for predicting rapid relapse. (v) The cancer tissue analysis tool had an accuracy of 70% for predicting relapse, and 80% for rapid relapse. (vi) The healthy tissue sample tool had an accuracy of 70% for relapse and rapid relapse, and (vii) the blood sample tool had an accuracy of 100% for relapse and 80% for rapid relapse.

This is but another way to examine PCa cells. It does pose several questions:

- 1. Pathways: Is there also a set of pathway malfunctions that one sees in PCa also present here?*

2. Is the CNV an artifact or causative. If causative then what is the specific process and how does it relate to known pathways.
3. This is a complex cellular measurement of genes. Is this cost effective?
4. The classic issue of stem cells again is raised. What chromosomes do we look at? Is this specific only to the PCa cells, the PCa stem cells, and all cells?

Definitions from Freeman et al:

1. Structural variant: A genomic alteration (e.g., a CNV, an inversion) that involves segments of DNA >1 kb.
2. Copy number variant (CNV): A duplication or deletion event involving >1 kb of DNA.
3. Duplicon :A duplicated genomic segment >1 kb in length with >90% similarity between copies
4. Indel: Variation from insertion or deletion event involving <1 kb of DNA.
5. Intermediate-sized structural variant (ISV): A structural variant that is ~8 kb to 40 kb in size. This can refer to a CNV or a balanced structural rearrangement (e.g., an inversion).
6. Low copy repeat (LCR): Similar to segmental duplication.
7. Multisite variant (MSV): Complex polymorphic variation that is neither a PSV nor a SNP.
8. Paralogous sequence variant (PSV): Sequence difference between duplicated copies (paralogs.)
9. Segmental duplication: Duplicated region ranging from 1 kb upward with a sequence identity of >90%. (Interchromosomal: Duplications distributed among nonhomologous chromosomes and Intrachromosomal: Duplications restricted to a single chromosome)
10. Single nucleotide polymorphism (SNP): Base substitution involving only a single nucleotide; ~10 million are thought to be present in the human genome at >1%, leading to an average of one SNP difference per 1250 bases between randomly chosen individuals

11.8 GENETIC SCREENING FOR PROSTATE CANCER

In a recent British Journal of Cancer article the authors have performed a preliminary analysis of genetic screening of those for higher risk for prostate and breast cancers. 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 does not readily allow direct and simple calculation from each other separately. We of course can extend this principle to n SNPs. It is obvious

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 \geq 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 then establishing a threshold one can bifurcate the groups. One could also select groups in some graded multi-sector grouping as well.

The SNPs chosen 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 ratio 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.

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 receive 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?

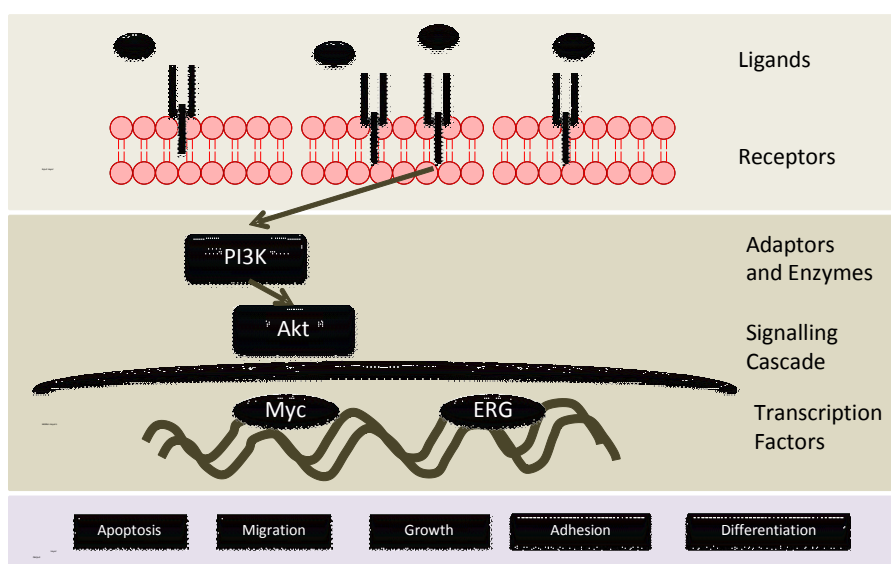
There are many more questions that arise from this work.

11.9 SUMMARY

It should be clear now that as we see the literature evolve we will undoubtedly have many new elements such that the picture will be continually be shuffled about. Yet it is essential that we keep anchored the issues of intracellular control and maintain a focus on what can be managed and what cannot. Our models will change incrementally each time we discover and add a new piece. The models must reflect reality in a predictive manner.

12 PATHWAY MODELS, VALIDATION AND CONTROLS

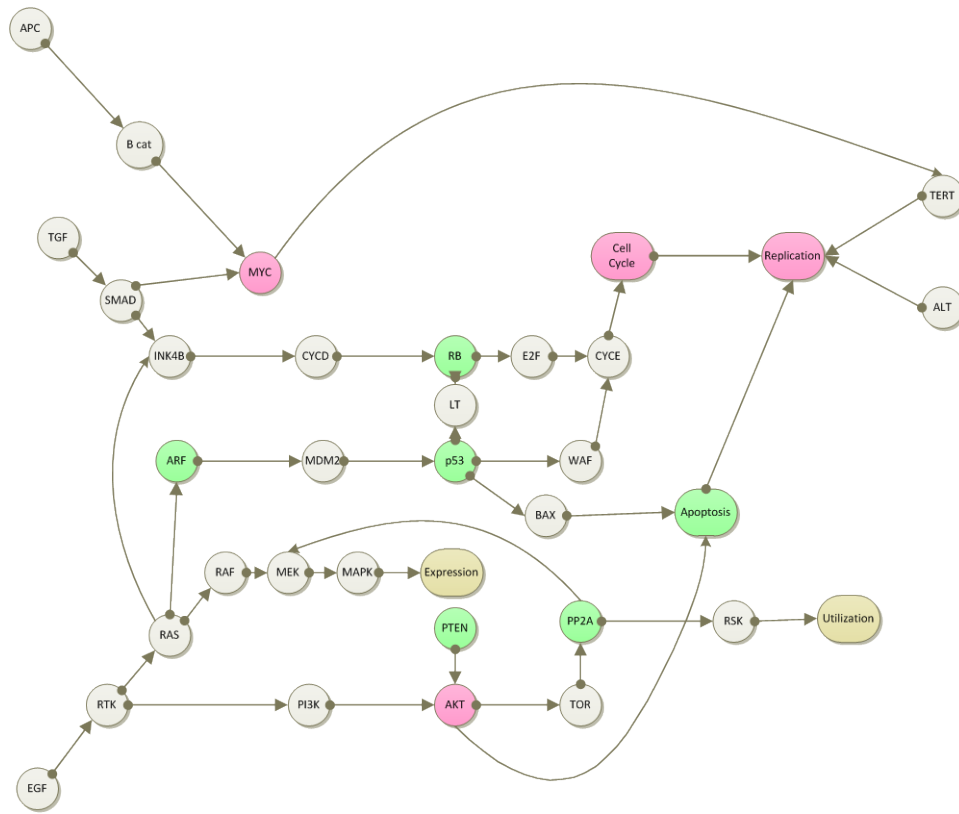
The pathway models are one in which one looks at a cell and examines what causes changes in the cell, growth or death, movement and relationship between cells. The figure below graphically depicts some of the key issues. The cell communicates to the outside via ligands and receptors, and these signals can then be transmitted to the cell nucleus via various chains of signalling mechanism. The signals effectors are most often proteins and these proteins result from genes being expressed in the nucleus. There are also proteins such as c-myc which depending on concentration can effect cell growth and replication, and there are the cyclin kinases which push the cell through the reproduction cycle.



The process can be viewed in two levels; normal cell metabolism of generating proteins via transcription and cell replication, namely the doubling of cells.

As a metric, there are 25 million cells in the human undergoing division per second. There are 50-100 trillion (million million) cells in the human body. Thus every second 0.5/million cells per second are dividing. Clearly that is not the case of every cell. Red blood cells live about 90 days and skin cells have a shorter lifetime. It is estimated that PCa cells double every two years but there can be a great deal of uncertainty in that number. For example if we have a PCa tumor mass of say 10 million cells, and we double to 20 million in 2 years that is 5 million in a year or about 2 cells per second. Recall that we have in the body a cell mitotic process of 25 million per second where a possible PCa is only a ten millionth of that number.

The current view of pathways is a connected map of interactions, activating and suppressing, resulting in certain actions by a cell. The typical example by Holmes is below. We have simplified it a bit.



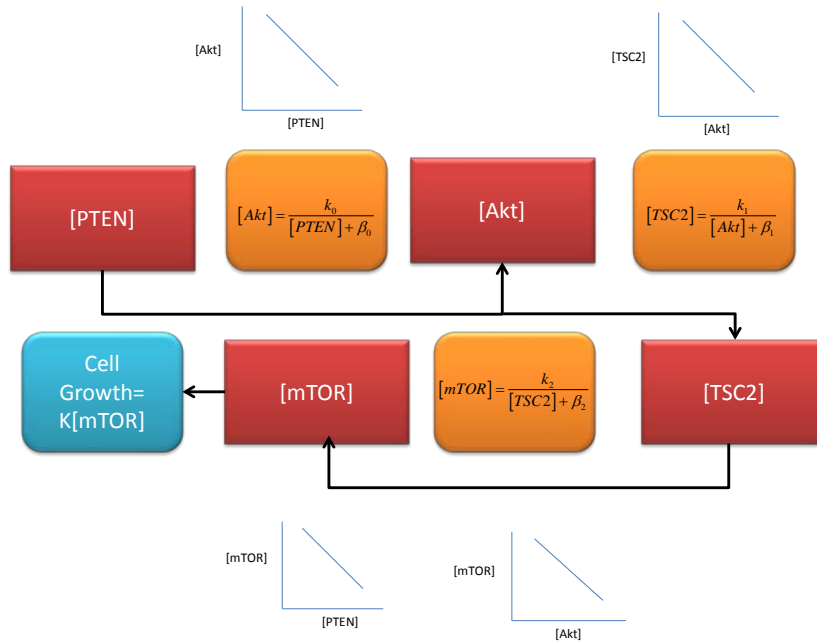
We have shown a few key genes and their products such as PTEN, MYC, p53, AKT and RB. The Holmes model is a graphical connective model and has been used by Milo, Shen-Orr and others. The issues we have with such is that it is necessary to have such a map yet the issues of concentrations, time, and feedback are missing. For example in the above we have PTEN sitting by its lonesome. One may ask what drives PTEN? Is its loss a total loss of the gene or a suppression or just a lowering of concentration with some complex mechanism of enzymatic reactions? To be able to answer these questions we clearly need a much more complex model.

As has been stated, we can summarize some of the key genes involved in PCa as follows:

| <i>Gene</i> | <i>Proposed function</i> |
|--|---|
| Mutations causing decreased activity | |
| <i>MS</i> | Anti-infectious, scavenger receptor |
| <i>RNASEL</i> | Anti-infectious, apoptosis |
| <i>ELAC2</i> | Metal-dependent hydrolase |
| Promoter hypermethylation resulting in gene silencing | |
| <i>GSTP1</i> | Carcinogen detoxification |
| Loss of heterozygosity and point mutation | |
| <i>PTEN</i> | Cell survival and proliferation |
| <i>TP53</i> (also <i>P53</i>) | Cell survival and proliferation, genome stability |
| Loss of heterozygosity and haplo insufficiency | |
| <i>NKX3-1</i> | Cell differentiation and proliferation |
| <i>CDKN1B</i> (<i>P27KIP1</i>) | Cell proliferation |
| Point mutations | |
| <i>COPEB</i> (also <i>KLR6</i>) | Transcription regulator |
| <i>AR</i> | Cell proliferation, survival, and differentiation |
| Amplification | |
| <i>AR</i> | Cell proliferation, survival, and differentiation |
| Overexpressed at mRNA and protein level | |
| <i>HTERT</i> | Cell immortality |
| <i>HPN</i> | Transmembrane protease |
| <i>FASN</i> | Fatty-acid synthesis |
| <i>AMACR</i> | Fatty-acid metabolism, branched chain |
| <i>EZH2</i> | Transcription repressor, cell proliferation |
| <i>MYC</i> | Cell proliferation |
| <i>BCL2</i> | Cell survival |
| Polymorphisms affecting prostate cancer risks | |
| <i>AR</i> | Cell proliferation, survival, and differentiation |
| <i>CYP17</i> | Androgen metabolism |
| <i>SRD5A2</i> | Androgen metabolism |

12.1 CELL GROWTH AND ITS METABOLIC CONTROL

One of the first elements of control is via the metabolic processes that control cell growth. It was in 1926 that Warburg proposed that cancer cells have a metabolic process which was anaerobic instead of aerobic like health cells. This premise then forced researchers to examine pathways which were significantly different than those in benign cells. A recent study by Levine and Kuter demonstrates in summary form how this applies to the cancer cells, many of which are core to PCa.



$$\frac{d[PTEN]}{dt} = \text{unknown}$$

$$\frac{d[Akt]}{dt} = f_1([PTEN], [Akt], t)$$

$$\frac{d}{dt} =$$

$$\frac{d[mTOR]}{dt} = f_3(t)$$

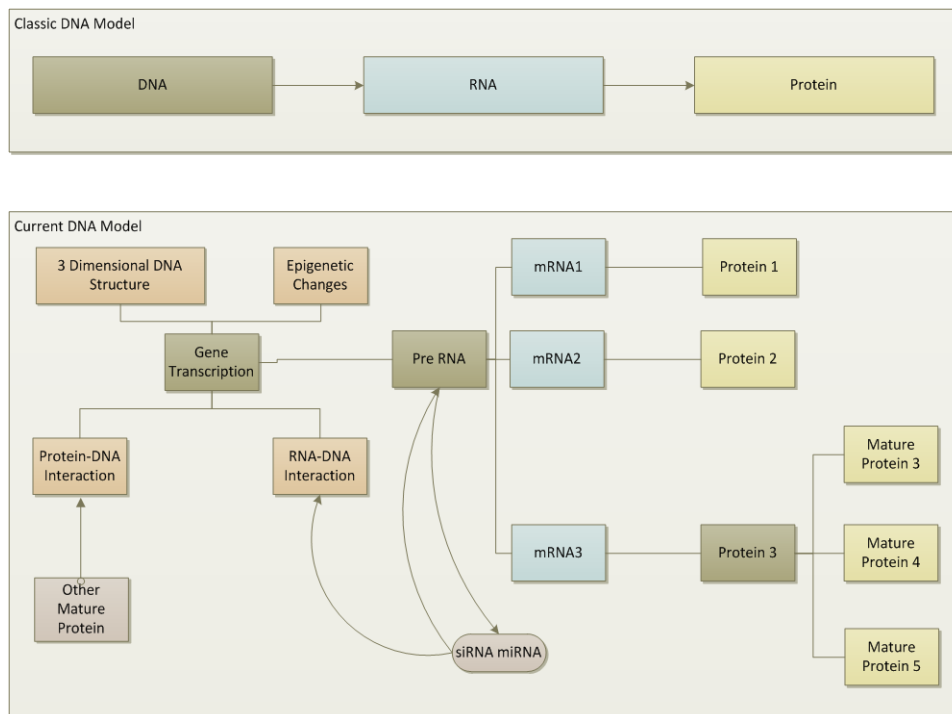
$$\frac{d[\text{Cell Growth}]}{dt} = g([mTOR])$$

We can expand this model depending on the complexity of the networks.

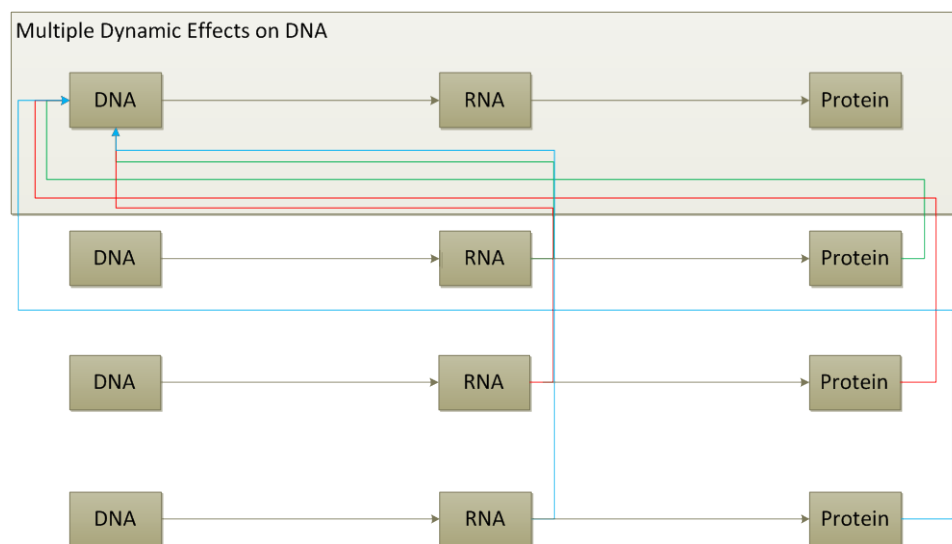
12.2 PATHWAY DYNAMICS

We now look at the evolution of pathways and how they work. Below we show a modified graphic based upon the work of Feero et al. On the top we show the classic Watson-Crick model. A simple progression from DNA to RNA to Protein. Then below we show how the concept has evolved to the way most see it today. Note that it still lacks the three factors we discussed earlier;

concentration, time factors, and full feedback. There is some feedback implied but a limited amount.



Now we can show some of the feedback mechanisms. This is in the graphic below:



Note that we have done this but for one gene segment. Remember here the DNA segment refers to a specific gene and the RNA the RNA resulting from that gene.

There are many models of pathway development and description. A simple approach is given in Bolouri which is basically an on-off type network switch design. He uses methods which have

some dynamics but is limited. In the book by Wang we find other similar alternatives. There is some excellent work in Szallasi et al and we have used it in the past. In McGarty (2008) we developed models which expanded the dynamics and allowed for the identification issue.

12.3 PATHWAYS, GENE EXPRESSION MEASUREMENT, METASTASIS

We have argued before that progress in cancer will develop as we better understand not just the histological appearance of the cells but the genetic expressions therein as well. Indeed many attempts at this have been taken and some seem to be bearing fruit. We look here at some recent work done at Dana Farber with regard to prostate cancer. The approach taken was to look at a large set of cells and then using massive microarray processing attempt to stratify the most significant markers related to metastasis. This is a useful example of understanding genes and their expression. However, our concern with their approach also highlights the overall lack of global knowledge and understanding of the genetic dynamics and linkages controlled by pathways. We use this example as a current step which explains the need for understanding the entire process and avoiding what Dougherty has commented on as the problem of just processing massive amounts of data and resolving on something which may or may not be the right thing.

One of the questions one frequently asks is how do we determine from a biopsy the eventual aggressiveness of PCa. This is a difficult question since we know for example that the loss of PTEN is often an ominous sign. It often pretends an already metastasized PCa, albeit without any way of determining where it has metastasized. In this section we look at a recent suggested test which would augment the histological analysis of Gleason scoring. The test proposed by the team at Dana Farber, of “Farber”, entails looking at gene profiles and then using them in a prognostic manner¹¹². We discuss this approach, which we have argued for in general before, and discuss its implications and present an alternative manner in which such tests in general may be analyzed.

The gene and gene products which were targeted are shown in the following Table:

| <i>Gene/Gene Product</i> | <i>Function</i> | <i>Location</i> |
|--------------------------|---|-----------------|
| <i>Pten</i> | <i>Pten controls the Akt pathway which if not controlled will lead to excessive cell growth.</i> | <i>10q23.3</i> |
| <i>Smad4</i> | <i>SMAD4 controls the G1 to S transition.</i> | <i>18q21.1</i> |
| <i>SPP1</i> | <i>SPP1 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.</i> | <i>4q21.1</i> |
| <i>CyclinD1</i> | <i>Cyclin D is a control with CDK4 and CDK6 of the transitions in the G1 to S stage of mitosis. Lack of control of Cyclin D will allow for uncontrolled cell growth.</i> | <i>11q13</i> |

We examine some of the issues related to this study and then discuss some new questions arising from it.

¹¹² <http://www.dana-farber.org/abo/news/press/2011/dana-farber-researchers-identify-molecular-predictor-of-metastatic-prostate-cancer.html>

Basically what this study has done is looked at the genomic content of a cell, a malignant cell, and it has tried to ascertain what the degree of potency for metastasis the cell may have. If it is an indolent cell then perhaps a wait and see attitude may prevail. If, however the profile indicates the potential for aggressive growth then surgery should be the option, or some other form of treatment eradicating the cells which hopefully are localized.

The approach by the researchers at the Farber seem to be to examine large samples and then using standard statistical techniques focus on a small targeted gene product set and if the expression of those genes is significantly over or under expressed then one can say with reasonable confidence that the aggressive treatment is warranted.

However this study does not seem to approach this study from a dynamical approach or an approach which relies on the essential pathways relating genes in the homeostasis of the cell.

12.3.1 Dana Farber Study

In the aforementioned recent Dana Farber research study the results state¹¹³:

“In the current study, researchers began with the well-established fact that prostate cancers without (sic) a working copy of the *Pten* gene tend to remain fairly idle and don't trespass beyond the prostate gland itself¹¹⁴. Researchers theorized that the loss of *Pten* in turn activates a collection of genes — a pathway — functioning to constrain the tumor's growth and invasion. If that pathway was shut down, they reasoned, the tumor would begin to break loose from the prostate and spread insidiously through the body.

Using computational biology techniques to analyze gene activity in mouse prostate cancer cells with inactive *Pten*, the investigators found a few pathways that seemed to play a constraining role. One, known as TGFβ-SMAD4 (for some of the genes that comprise it), was particularly intriguing as this pathway had been implicated in the metastasis of other tumor types in the past. When researchers conducted confirmatory molecular signaling studies to see what happens when *Pten* is knocked out of commission, signaling in the TGFβ-SMAD4 pathway "shot through the roof," DePinho says, suggesting that the pathway had sprung into action.

When researchers generated mice whose prostate cells lacked both *Pten* and the *Smad4* gene, the animals developed large, fast-growing tumors that spread to their lymph nodes and beyond. Guided by these insights, they then examined whether something similar was happening in human prostate cancers.

¹¹³ <http://www.dana-farber.org/abo/news/press/2011/dana-farber-researchers-identify-molecular-predictor-of-metastatic-prostate-cancer.html> also see Nature paper <http://www.nature.com/nature/journal/vaop/ncurrent/full/nature09677.html>

¹¹⁴ We believe that this is a mis-statement. PTEN inactivation is known in metastatic PCa and thus we suspect that they are misquoted. The remainder of the article enforces this belief.

Comparing the gene expression profiles of indolent versus aggressive mouse prostate cancers, they found about 300 genes that distinguished the two groups. "We then categorized them for known functions," DePinho says. "We were encouraged to see that the top functional category were genes playing that have roles in cell division and movement" — actions that are needed for cancer cells to grow and spread with lethal consequences.

The researchers conducted an elaborate series of experiments to identify the genes most closely linked to the aggressive biology of prostate cancer. Among the hundreds of genes analyzed, two such genes stood out: *SPPI* and *CyclinD1*, both of which, intriguingly, are close working partners of *Smad4*.

The four-gene signature — *Pten*, *Smad4*, *SPPI*, and *CyclinD1* — showed its effectiveness as a predictive tool for survival when researchers drew on data from the Physicians' Health Study, which has been tracking the health of thousands of U.S. physicians for nearly 30 years. When the investigators screened prostate cancer samples from study participants for the four-gene/protein signature, it was more accurate in predicting the ultimate course of the illness than conventional methods were.

"By integrating a variety of techniques — computational biology, genetically engineered model systems, molecular and cellular biology, and human tissue microarrays — we've identified a signature that has proven effective in distinguishing which men with prostate cancer are likely to progress and die from their disease and those who are not," DePinho remarks. "Efforts are already underway to use this knowledge to develop a clinical test — which we hope will occur within a year or so — that will enable doctors and patients to make more accurate treatment decisions and avoid unnecessary aggressive interventions which adversely impact on quality of life and deplete over-extended healthcare resources. This science holds potential to illuminate a long-sought answer for optimal management of this complex disease."

Thus we look again at the pathways. Our interest is in those pathways which effect:

1. *Pten*,
2. *Smad4*,
3. *SPPI*, and
4. *CyclinD1*

12.3.2 PTEN Suppression

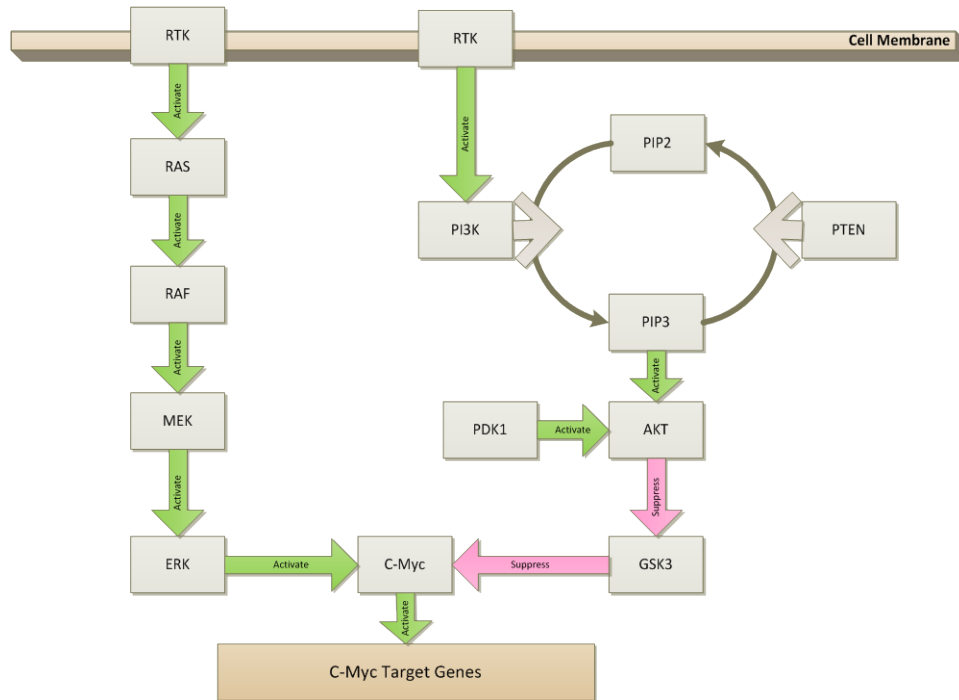
PTEN is a significant gene which controls the Akt pathway which in turn controls the replication of cells. Loss of PTEN is often seen in metastatic prostate cancer. In many ways it is the hallmark of this change. As stated in NCBI¹¹⁵:

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,

¹¹⁵ <http://www.ncbi.nlm.nih.gov/gene/5728>

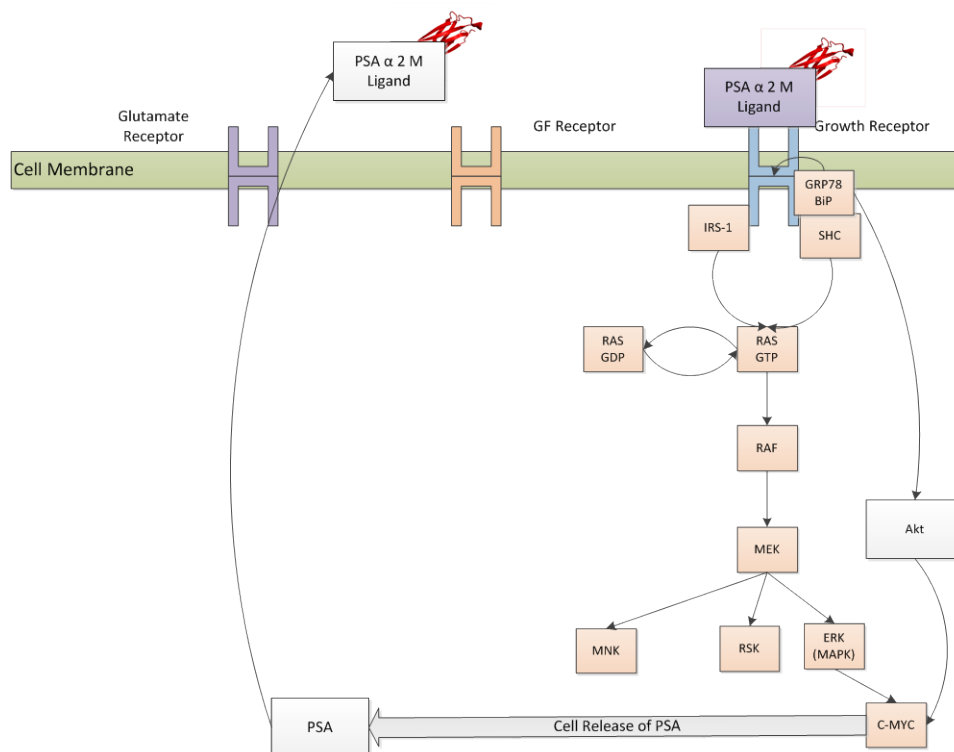
this protein preferentially dephosphorylates phosphoinositide substrates. It negatively regulates intracellular levels of phosphatidylinositol-3,4,5-trisphosphate in cells and functions as a tumor suppressor by negatively regulating AKT/PKB signaling pathway.

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.

We can amend this with the details on the Ras pathway as shown below:



12.3.3 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 kinase or CDK. The activated cyclin-CDK complex phosphorylates 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.

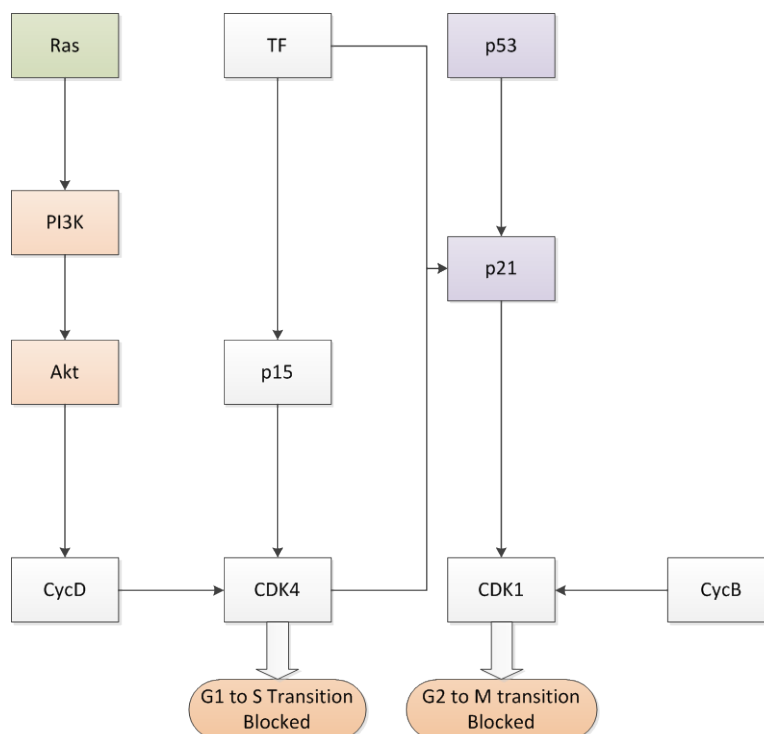
As stated in NCBI¹¹⁶:

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.

¹¹⁶ <http://www.ncbi.nlm.nih.gov/gene/595>

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

Now we can look more closely at Cyclin D, CycD, as we show below. This we show as follows:



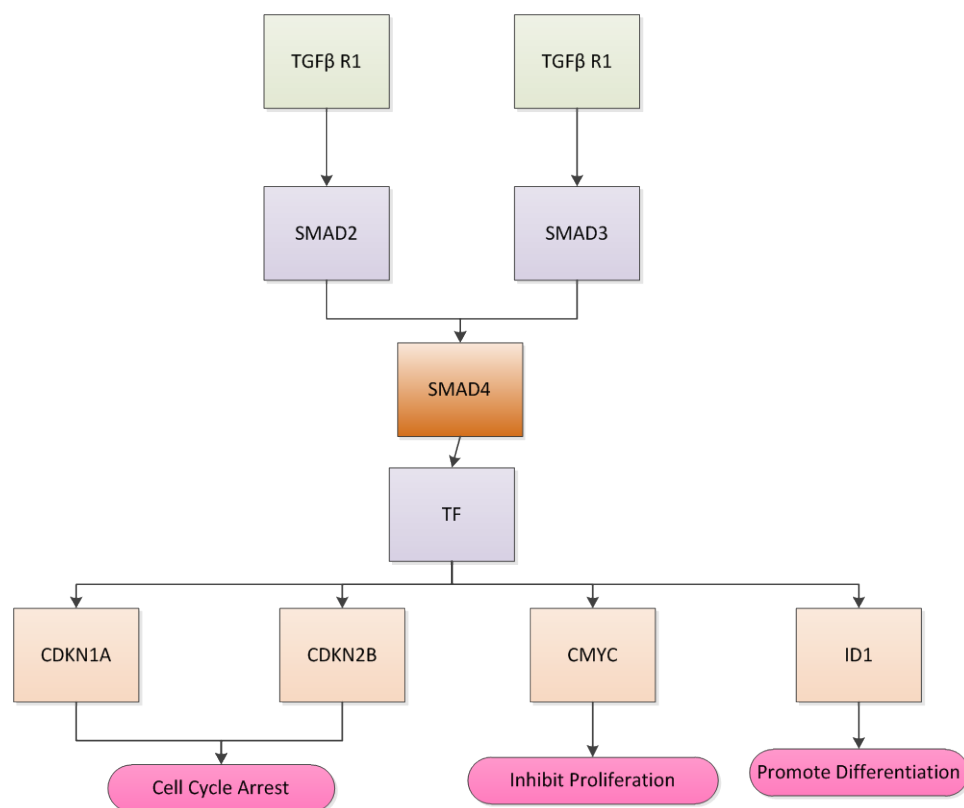
Note that Cyc D if not regulated will in turn fail to regulate the blocking of the G1 to S transition.

12.3.4 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 NCBI¹¹⁷:

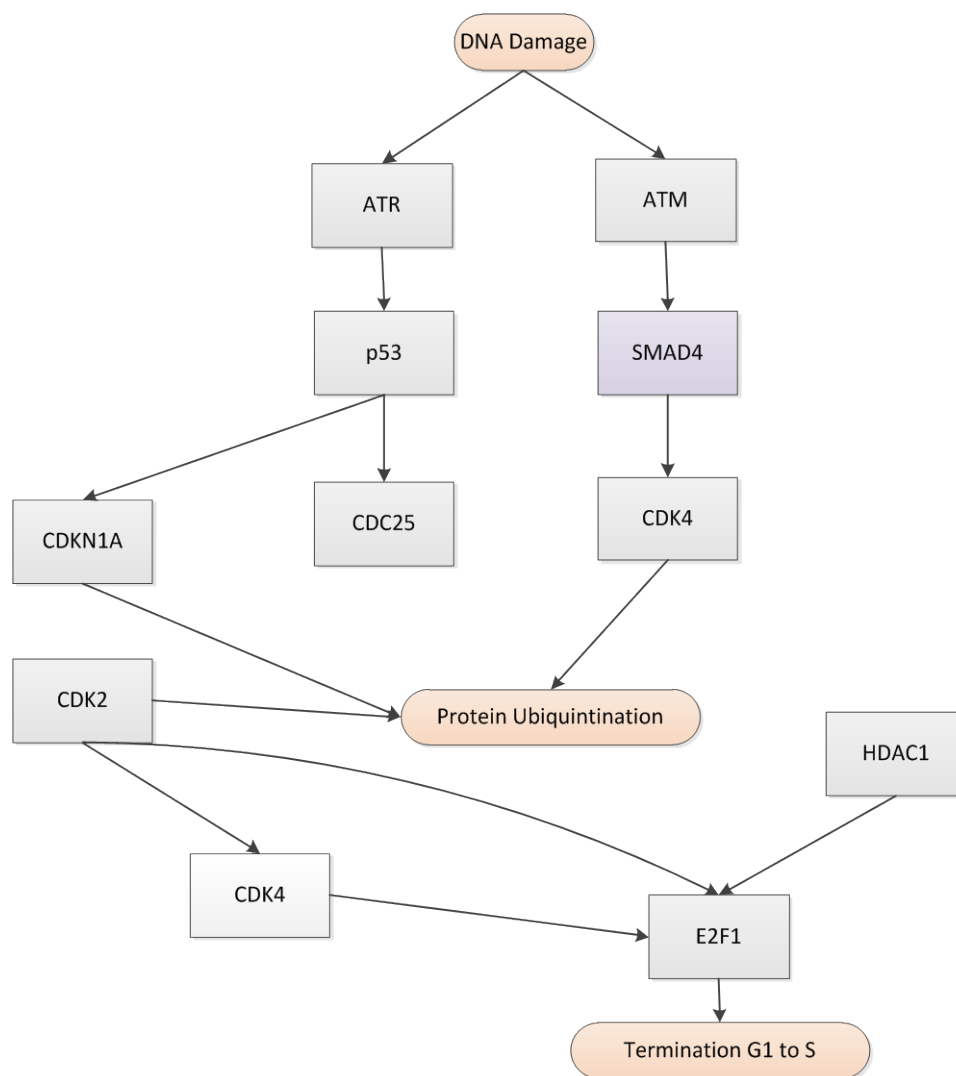
This gene encodes a member of the Smad family of signal transduction proteins. Smad proteins are phosphorylated and activated by transmembrane serine-threonine receptor kinases in response to TGF-beta signaling. The product of this gene forms homomeric complexes and heteromeric complexes with other activated 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 pathway¹¹⁸:

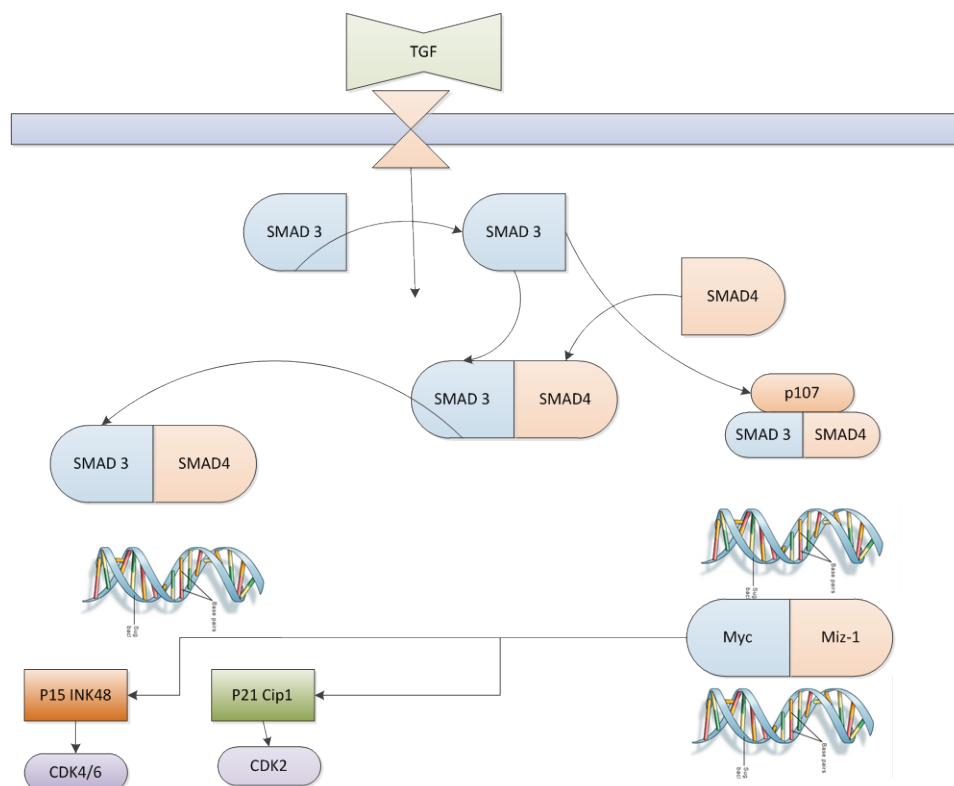
¹¹⁷ <http://www.ncbi.nlm.nih.gov/gene/4089>

¹¹⁸ 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.

12.3.5 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 species¹¹⁹.

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.

¹¹⁹ Also see <http://www.ncbi.nlm.nih.gov/gene/6696> also see <http://www.wikigenes.org/e/gene/e/6696.html>

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.

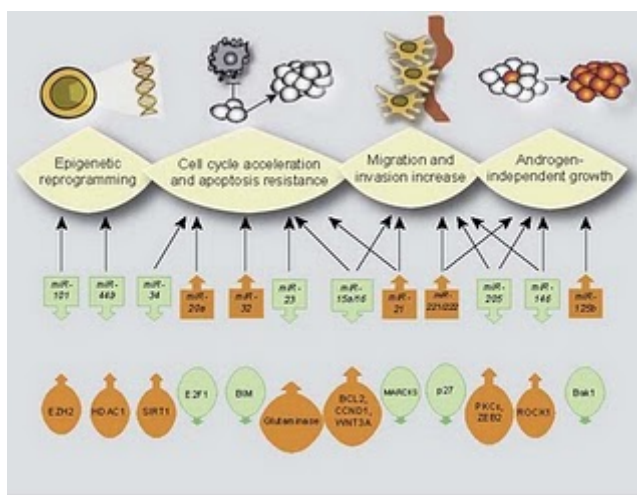
SPP1 is a SIBLING glycoprotein that was first identified in osteoblasts. OPN is an important anti-apoptotic factor in many circumstances. OPN blocks the activation-induced cell death of macrophages and T cells as well as fibroblasts and endothelial cells exposed to harmful stimuli. OPN prevents non-programmed 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.

12.3.6 Analysis

In a recent announcement from [Dana Farber](#) in Boston, a paper has been prepared that indicates that testing for four gene products significantly improves the ability to determine an indolent Prostate Cancer from an aggressive form. The results also hit the news including a [WSJ](#) release.

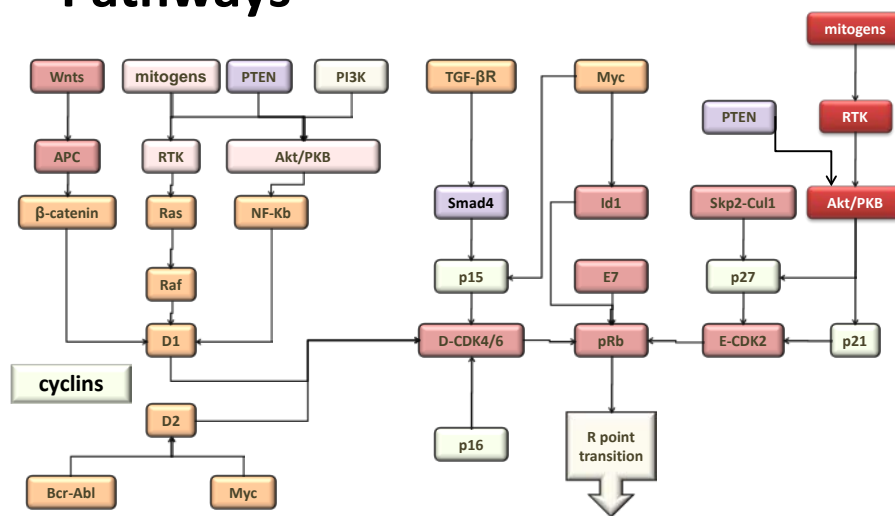
The release from DF states:

The four-gene signature — Pten, Smad4, SPP1, and CyclinD1 — showed its effectiveness as a predictive tool for survival when researchers drew on data from the Physicians' Health Study, which has been tracking the health of thousands of U.S. physicians for nearly 30 years. When the investigators screened prostate cancer samples from study participants for the four-gene/protein signature, it was more accurate in predicting the ultimate course of the illness than conventional methods were.



We show some of the pathway elements above. A more detailed version is below.

Pathways



23

In the above we show the PTEN control, the Smad4 control and the presence of the cyclins. The loss of PTEN has been known for years to be a sign that metastasis may very well already started. SMAD4, SPP1 and the Cyclin D1 are in pathways that also control the growth of the cell. PTEN is most often the one pathway element of most concern.

The driving factor in the result is:

The standard measure of prostate cancer's aggressiveness, known as the Gleason score (which is based on cancer cells' appearance under a microscope), is accurate about 60 to 70 percent of the time depending on the skill of the pathologist. The four-gene signature method alone was accurate 83 percent of the time. Combining the markers and Gleason methods produced an accuracy of approximately 90 percent.

Now the above result need some clarification.

1. Gleason scores are score based upon the histological presentation of the cells. Gleason 1 for example is low grade and shows the cells as small gland like structures but lacking gland architecture. Gleason 5 is a mass of undifferentiated cells clustered about the stroma, internal part of the prostate, with no structure. Gleason scores are the sum of the most prevalent cell type plus the next most prevalent so a 7 is a 4+3 or a 3+4. Clearly a 4+3 is more severe than a 3+4. But Gleason alone tells one little about the metastatic potential.

2. Genetic pathway changes are often the sine qua non for determination. But what genes and in what cells. The problem is the existence of the cancer stem cell idea, namely that one of the many cancer like cells is pluripotent and if this is true in prostate cancer then it is that cell we want. So perhaps in a biopsy we missed the killer cell, or it may have already escaped.

3. This procedure statistically takes us a step forward, now we can test to see if we do have some very bad cells. However one may ask if we are finding out when the cow has already left the barn. Namely what we may have is a test which confirms the fact that the patient's cancer is one of those very bad types so nothing much can be done. On the other hand we may have a patient with an aggressive form which is just a threshold below the bad level. Thus one need significant clinical tests.

4. The question then is; are there other pathway constituents that are prognostic enough to have a meaningful result in mortality, namely how well do we know the PCa pathways. What are the pathway dynamics between these four, we know some, and many are available through [NCI](#).

5. The results appear to have quite extensive, but causality and dynamics still need some filling in. What caused the change. In addition one may look at the HGPIN cases and see that we have HGPIN which all too often is considered as pre-malignant, but we know that HGPIN also regresses to a fully benign prostate. What happened.

The questions that seem to remain all relate to pathway dynamics. They are:

1. What are the pathway dynamics of each of these markers. Is there some causal relationship or are they independent events?
2. What caused the change in expression? Is there a change in the gene or are there other factors. Are there epigenetic issues such as miRNA or methylation. Are there activator or repressor genes related to the transcription of these genes which result in the change. If so how are these genes related in the overall gene network.
3. What are the temporal issues of this gene network? Which changes are causal and which are simultaneous.
4. What are the spatial issues, namely do the modified cells communicates with others to allow for the development of PCa clusters and if so how do these genes function in such an environment.
5. What of the stem cell issue? If there are PCa stem cells and these cells are drivers of the overall metastatic process, does sampling for these markers reflect the stem cell or the tumor mass in general? The CSC is a significant question and if one must select that cell how does one identify the cell? Are there CSC markers for CSC PCa cells.

These are but a few of the questions. The lingering one however is when these markers are detected is it really one where the cow is out of the barn or is it just time to bar the door?

As the WSJ states:

Dr. DePinho said the new study suggests a test based on the four genes, when added to current prediction protocols could improve the accuracy to about 90%....Charles Sawyers, a cancer expert and Howard Hughes Medical Institute Investigator at Memorial Sloan Kettering Cancer

Center in New York, said if it turns out that such a test is that effective, "it would have a huge impact." He wasn't involved in this research.

Dr. Sawyers cautioned that such gene signatures have been proposed to predict cancer progression in the past and haven't panned out. He said the genes the new research focuses on appear to have a functional role in regulating cancer development, but that more research will be necessary to validate whether a test would have value for doctors and patients... "We'll have to wait and see," he said.

Indeed one must wait and see. However this will be a challenge to Medicare, since so many of the patients are Medicare patients. How effective is this test? If the genes are all showing aggressive growth then we act? Or is it too late already. If the genes do not show aggressive growth do we deny the patient care even if the patient has strong family history of aggressive PCa? The issue is that all too often the family history dominates. Perhaps this creates more issues than it solves, however it is a step in a good direction, it may or may not be the right one.

12.3.7 Gene Tables

The following Tables are taken from the paper by Chandran et al and represent a significant amount of detail on all genes which have been identified as either over or under expressed in PCa. Causality is not evident.

Table 3: Transcripts with median values with at least 3 fold difference between metastatic and primary tumor samples

| Gene Symbol | Probe_ID | P1 | P2 | P3 | P4 |
|-------------|------------|-------|-------|-------|--------|
| HBB | 32052_at | 22.37 | 5.78 | 13.25 | 56.28 |
| SPP1 | 34342_s_at | 24.16 | 26.78 | 4.75 | 5.39 |
| HBA1///HBA2 | 31525_s_at | 15.14 | 4.47 | 13.65 | 108.11 |
| LGR4 | 43585_at | 7.39 | 7.43 | 20.89 | 24.82 |
| AR | 1577_at | 14.35 | 12.97 | 12.24 | 14.78 |
| PRO1073 | 49666_s_at | 4.56 | 13.25 | 10.01 | 13.50 |
| UTRN | 42646_at | 10.11 | 6.02 | 12.11 | 16.31 |
| HNT | 59070_at | 5.37 | 9.69 | 12.08 | 13.67 |
| SDCCAG3 | 43014_at | 7.99 | 8.57 | 11.24 | 17.32 |
| LOC64744 | 42739_at | 7.30 | 9.64 | 9.57 | 14.51 |
| --- | 1089_i_at | 5.06 | 4.14 | 12.12 | 22.01 |
| SPP1 | 2092_s_at | 14.05 | 12.94 | 3.35 | 4.07 |
| UBE2H | 58777_at | 9.50 | 7.45 | 6.55 | 15.13 |
| SRPK1 | 63687_at | 6.06 | 4.36 | 10.61 | 12.82 |
| NCK2 | 33003_at | 5.00 | 9.14 | 8.50 | 7.34 |
| HIST1H3H | 36757_at | 7.26 | 17.07 | 8.47 | 5.61 |
| PPP4R2 | 48663_at | 5.09 | 6.97 | 8.59 | 16.16 |
| C8orf16 | 47339_at | 6.54 | 8.15 | 9.53 | 7.39 |
| --- | 55943_at | 3.41 | 7.47 | 15.31 | 8.01 |
| --- | 64642_s_at | 8.25 | 6.42 | 7.04 | 10.60 |
| EP400 | 47518_at | 5.94 | 9.27 | 4.51 | 9.32 |
| GOLT1A | 45144_at | 3.90 | 6.17 | 8.37 | 12.32 |
| --- | 52853_g_at | 9.83 | 7.10 | 6.25 | 7.03 |
| LOC284058 | 44791_at | 8.25 | 10.17 | 4.55 | 5.86 |
| DAPK1 | 51580_at | 3.42 | 6.04 | 8.03 | 11.32 |
| NFATC2IP | 38864_at | 3.26 | 4.83 | 9.58 | 9.19 |
| SEL1L | 40689_at | 4.71 | 7.84 | 6.13 | 10.94 |
| TM4SF9 | 47746_at | 3.43 | 6.26 | 8.92 | 7.52 |
| MLLT2 | 65205_at | 3.43 | 7.13 | 6.57 | 13.01 |
| SC4MOL | 46802_at | 22.91 | 7.35 | 5.62 | 6.17 |
| --- | 62671_at | 6.38 | 7.13 | 5.74 | 11.03 |
| BIRC6 | 46558_at | 5.67 | 8.59 | 7.50 | 5.92 |
| MAP4K4 | 51474_at | 4.86 | 4.32 | 8.70 | 8.52 |
| MLLT2 | 53300_at | 4.65 | 3.99 | 9.39 | 8.10 |
| --- | 52851_at | 8.71 | 5.94 | 6.35 | 6.25 |
| MRRF | 51635_at | 4.23 | 4.87 | 7.39 | 8.23 |
| ACAS2 | 62783_at | 4.29 | 6.14 | 7.02 | 5.90 |
| --- | 60658_at | 3.40 | 6.84 | 5.19 | 9.10 |
| SUMO1 | 49551_at | 4.05 | 7.20 | 4.77 | 7.75 |
| AR | 1578_g_at | 7.56 | 4.86 | 5.32 | 6.37 |
| GALNT7 | 59101_at | 8.41 | 4.12 | 5.11 | 6.54 |
| GPR75 | 44203_at | 5.14 | 8.32 | 3.90 | 6.31 |
| TBL1XR1 | 65001_r_at | 3.53 | 12.30 | 4.06 | 7.19 |

| | | | | | |
|-----------|------------|-------|-------|-------|-------|
| HSD17B12 | 43292_at | 4.74 | 8.88 | 3.64 | 6.28 |
| MRPS28 | 43095_at | 5.79 | 5.39 | 5.58 | 5.14 |
| FN1 | 64719_at | 27.07 | 6.02 | 4.05 | 4.93 |
| GPR158 | 44214_at | 7.21 | 3.33 | 4.32 | 6.62 |
| --- | 48069_at | 6.27 | 9.88 | 3.38 | 4.55 |
| FLJ21657 | 58778_at | 4.34 | 5.60 | 6.17 | 5.18 |
| MLL5 | 43301_at | 4.76 | 3.61 | 5.87 | 10.34 |
| --- | 55761_at | 3.78 | 4.88 | 5.65 | 6.93 |
| DLG1 | 47231_at | 3.40 | 4.77 | 6.22 | 5.70 |
| MYO5B | 63281_r_at | 3.29 | 6.17 | 4.29 | 6.84 |
| --- | 49268_at | 3.55 | 19.86 | 3.61 | 6.75 |
| FUS | 43501_at | 3.93 | 3.78 | 6.42 | 8.97 |
| CCDC35 | 54684_at | 4.90 | 8.14 | 3.55 | 5.43 |
| --- | 43435_at | 6.85 | 4.83 | 4.82 | 5.49 |
| SMA4 | 32921_at | 4.68 | 5.53 | 5.74 | 4.26 |
| NCOA1 | 45953_at | 6.53 | 4.13 | 3.58 | 6.06 |
| S100A8 | 41096_at | 4.22 | 5.89 | 3.80 | 22.58 |
| PRKCBP1 | 53493_at | 4.65 | 7.37 | 4.50 | 5.35 |
| RNPC2 | 65083_at | 3.18 | 3.96 | 6.01 | 9.19 |
| CAMSAP1 | 62630_at | 4.45 | 5.80 | 3.36 | 5.36 |
| EEF1G | 41903_at | 5.19 | 4.58 | 4.31 | 5.34 |
| EIF5 | 51379_at | 3.44 | 4.08 | 5.62 | 11.07 |
| MAML3 | 49879_at | 3.39 | 3.22 | 10.27 | 5.87 |
| C21orf106 | 59651_at | 3.19 | 4.02 | 5.23 | 6.44 |
| VCIP135 | 42715_at | 3.37 | 3.61 | 5.52 | 8.55 |
| FOXO3A | 55502_at | 3.48 | 4.37 | 6.97 | 4.74 |
| C7orf20 | 49143_s_at | 4.23 | 4.62 | 4.41 | 5.78 |
| GNMT | 46482_at | 3.59 | 4.84 | 4.24 | 4.64 |
| DONSON | 48549_at | 4.10 | 3.58 | 4.66 | 5.28 |
| --- | 43436_g_at | 4.98 | 3.75 | 3.58 | 5.09 |
| PKP4 | 66327_at | 3.31 | 3.88 | 4.56 | 6.20 |
| PCBP2 | 55393_at | 3.73 | 3.19 | 4.36 | 6.29 |
| CPEB4 | 57169_at | 3.70 | 3.92 | 4.14 | 4.48 |
| CUGBP1 | 34683_at | 4.26 | 3.76 | 3.13 | 4.78 |
| FALZ | 47458_at | 4.21 | 3.65 | 3.82 | 4.09 |
| --- | 51586_at | 3.51 | 4.00 | 4.99 | 3.89 |
| RALA | 39253_s_at | 3.92 | 4.30 | 3.29 | 3.85 |
| MLL5 | 45092_at | 4.36 | 3.21 | 4.48 | 3.39 |
| PABPC1 | 44806_at | 3.74 | 3.98 | 4.20 | 3.07 |
| EIF1AX | 34278_at | 3.99 | 3.47 | 3.84 | 3.19 |
| C7orf2 | 42173_at | 3.15 | 3.27 | 5.07 | 4.04 |
| --- | 63147_at | 3.25 | 5.40 | 3.12 | 4.04 |
| RAD23B | 41157_at | 3.20 | 3.46 | 3.64 | 4.45 |
| --- | 61037_at | 3.44 | 3.56 | 3.47 | 3.73 |
| NFATC1 | 39143_at | 3.13 | 3.21 | 9.06 | 3.78 |
| JARID1A | 50532_at | 3.22 | 3.32 | 3.54 | 4.12 |
| PDLIM5 | 37366_at | 3.02 | 3.58 | 3.42 | 3.16 |

Table 3: Transcripts with median values with at least 3 fold difference between metastatic and primary tumor samples

| Gene Symbol | Probe_ID | P1 | P2 | P3 | P4 |
|---------------|------------|----------|----------|---------|---------|
| NEFH | 33767_at | (117.15) | (147.36) | (9.90) | (17.18) |
| C10orf116 | 32527_at | (35.49) | (29.63) | (46.85) | (66.50) |
| KLK11 | 40035_at | (23.65) | (19.24) | (39.73) | (62.15) |
| FAM3B | 59657_at | (15.81) | (27.92) | (26.09) | (25.97) |
| PGM5 | 52140_at | (23.87) | (26.50) | (44.27) | (17.72) |
| MRGPRF | 52946_at | (15.61) | (18.57) | (30.59) | (70.95) |
| KRT15 | 37582_at | (21.85) | (20.74) | (19.22) | (33.68) |
| PTN | 34820_at | (11.62) | (31.95) | (10.24) | (27.11) |
| SELM | 64449_at | (6.36) | (8.40) | (29.23) | (39.36) |
| MYLK | 46276_at | (5.87) | (15.22) | (22.57) | (20.86) |
| SYNPO2 | 50361_at | (15.14) | (15.77) | (20.15) | (84.14) |
| KRT5 | 613_at | (13.21) | (11.12) | (22.66) | (32.96) |
| FOS | 2094_s_at | (10.72) | (25.75) | (13.72) | (16.45) |
| PKP1 | 51214_at | (11.57) | (16.34) | (11.83) | (17.85) |
| --- | 42921_at | (9.96) | (11.67) | (15.61) | (16.50) |
| RAB34 | 45269_at | (14.36) | (11.54) | (17.49) | (10.35) |
| --- | 48927_at | (10.61) | (14.93) | (8.77) | (21.91) |
| ALOX15B | 37430_at | (12.47) | (12.41) | (14.17) | (9.10) |
| FOS | 1915_s_at | (7.59) | (26.38) | (11.03) | (12.11) |
| TMEM16G | 62387_at | (9.63) | (13.32) | (12.59) | (9.93) |
| --- | 64676_at | (17.30) | (9.39) | (6.32) | (13.05) |
| SFRP1 | 32521_at | (13.10) | (5.73) | (8.29) | (16.73) |
| NDFIP2 | 60510_at | (7.20) | (9.23) | (11.72) | (15.15) |
| FHOD3 | 50298_at | (9.96) | (12.84) | (5.59) | (10.96) |
| WNT5B | 61292_s_at | (8.72) | (11.85) | (5.42) | (13.92) |
| SYNPO2 | 48039_at | (11.04) | (8.80) | (12.64) | (9.34) |
| BOC | 64423_s_at | (3.63) | (8.16) | (11.80) | (54.66) |
| SLC20A2 | 1137_at | (9.27) | (5.08) | (10.51) | (12.61) |
| COL8A2 | 52652_g_at | (7.95) | (9.99) | (11.56) | (9.75) |
| --- | 52678_at | (9.69) | (9.99) | (3.76) | (17.93) |
| FOS | 1916_s_at | (7.58) | (21.81) | (6.93) | (11.58) |
| ARGBP2 | 51939_at | (7.77) | (13.86) | (10.40) | (8.71) |
| CTGF | 64342_at | (4.21) | (4.15) | (20.44) | (14.87) |
| EPHB6 | 39930_at | (8.61) | (9.66) | (8.32) | (19.41) |
| SYNPO2 | 60532_at | (9.77) | (5.54) | (8.77) | (9.03) |
| NR4A1 | 280_g_at | (8.68) | (13.49) | (5.82) | (8.58) |
| DKFZP564O0823 | 54033_at | (4.67) | (3.72) | (11.83) | (20.00) |
| GSTO2 | 45609_at | (4.73) | (6.81) | (9.60) | (16.18) |
| --- | 49321_at | (7.91) | (8.41) | (9.24) | (3.88) |
| EGR3 | 40375_at | (9.89) | (7.71) | (8.49) | (6.44) |
| SYNPO2 | 61681_at | (7.85) | (8.33) | (4.56) | (18.57) |

| | | | | | |
|---------------|------------|---------|---------|---------|---------|
| PI15 | 58361_at | (3.59) | (4.26) | (12.77) | (11.74) |
| FOSB | 36669_at | (8.81) | (6.27) | (7.60) | (8.39) |
| OGN | 43507_g_at | (3.56) | (8.26) | (7.19) | (25.54) |
| MOXD1 | 36834_at | (5.40) | (11.70) | (10.00) | (3.85) |
| LSAMP | 43930_at | (3.05) | (7.62) | (9.76) | (7.67) |
| EGR2 | 37863_at | (7.70) | (5.52) | (7.23) | (15.41) |
| DKFZp686D0853 | 49770_at | (10.18) | (7.66) | (7.16) | (4.39) |
| LGP1 | 52826_at | (13.75) | (5.94) | (3.83) | (8.11) |
| ME3 | 35216_at | (7.45) | (9.26) | (6.54) | (5.32) |
| PPP1R14A | 58774_at | (6.68) | (6.14) | (7.31) | (7.87) |
| FLJ22386 | 50198_at | (6.80) | (3.64) | (6.98) | (6.65) |
| NR4A1 | 279_at | (5.31) | (8.04) | (5.11) | (8.48) |
| WFDC1 | 64111_at | (3.79) | (11.21) | (6.64) | (6.66) |
| ZFP36 | 40448_at | (6.39) | (6.86) | (7.25) | (3.61) |
| CACHD1 | 43554_at | (6.68) | (3.34) | (17.46) | (6.57) |
| RLN1 | 35070_at | (6.78) | (11.78) | (5.14) | (6.39) |
| --- | 49975_at | (6.43) | (6.16) | (6.74) | (10.11) |
| CYBRD1 | 65852_at | (6.43) | (4.79) | (6.70) | (7.23) |
| PER3 | 53766_at | (15.43) | (6.79) | (5.56) | (6.29) |
| MN1 | 37283_at | (4.47) | (7.36) | (5.55) | (7.48) |
| DNCI2 | 35788_at | (4.20) | (8.68) | (3.02) | (10.64) |
| MRVI1 | 43966_at | (6.76) | (5.28) | (12.19) | (6.09) |
| AZGP1 | 35834_at | (6.32) | (3.86) | (38.18) | (6.18) |
| MGC14839 | 48949_at | (8.96) | (4.19) | (8.25) | (3.61) |
| SMTN | 64499_s_at | (5.20) | (15.22) | (7.18) | (4.42) |
| HSPC157 | 50179_at | (5.66) | (3.18) | (6.63) | (8.09) |
| WFDC2 | 33933_at | (5.30) | (6.50) | (5.73) | (6.81) |
| BTG2 | 36634_at | (6.99) | (3.13) | (9.25) | (5.22) |
| AXIN2 | 64129_at | (4.97) | (6.97) | (7.18) | (4.20) |
| PDGFC | 45217_at | (4.32) | (7.53) | (8.81) | (3.97) |
| MLLT10 | 63345_at | (7.20) | (5.85) | (5.90) | (3.84) |
| BMP7 | 49273_g_at | (4.58) | (4.89) | (6.82) | (13.13) |
| MCC | 49504_r_at | (5.90) | (5.71) | (5.08) | (5.84) |
| HEXA | 39340_at | (8.15) | (5.65) | (4.18) | (5.88) |
| GSTT2 | 1099_s_at | (6.47) | (5.05) | (6.80) | (4.66) |
| SSPN | 65647_at | (5.40) | (5.88) | (3.12) | (17.61) |
| UPK3A | 36379_at | (5.37) | (4.71) | (5.81) | (6.91) |
| PDE5A | 54668_at | (4.44) | (5.17) | (5.87) | (9.56) |
| PSD3 | 63832_at | (3.19) | (6.04) | (4.98) | (6.58) |
| ALDH7A1 | 61965_at | (5.85) | (5.14) | (5.88) | (3.13) |
| FMOD | 33431_at | (7.62) | (4.30) | (4.90) | (6.04) |
| TSPAN2 | 53693_at | (6.38) | (4.49) | (4.54) | (6.77) |
| DKFZP586H2123 | 40017_at | (6.52) | (6.49) | (4.32) | (3.91) |
| EFS | 33883_at | (5.43) | (3.58) | (6.35) | (5.18) |
| PODN | 63953_at | (4.16) | (5.30) | (4.84) | (4.98) |
| DUSP1 | 1005_at | (6.53) | (16.66) | (3.02) | (3.19) |
| SLC22A17 | 58898_s_at | (4.93) | (5.81) | (4.66) | (4.44) |

| | | | | | |
|---------------|------------|--------|--------|--------|---------|
| CDH10 | 47535_at | (4.87) | (3.19) | (8.27) | (4.65) |
| --- | 64163_at | (3.66) | (5.03) | (4.79) | (4.70) |
| --- | 42587_at | (4.68) | (4.62) | (4.90) | (3.45) |
| TSPAN2 | 57331_at | (4.44) | (8.06) | (3.42) | (4.71) |
| SORBS1 | 56409_at | (5.45) | (5.70) | (3.17) | (3.53) |
| C21orf63 | 50658_s_at | (4.54) | (3.36) | (4.15) | (5.31) |
| NBL1 | 37005_at | (3.34) | (4.27) | (4.31) | (6.36) |
| CIRBP | 39864_at | (4.38) | (3.53) | (4.19) | (6.80) |
| KLF4 | 48587_at | (3.77) | (3.62) | (4.57) | (12.50) |
| ZCSL2 | 45320_at | (3.10) | (3.19) | (5.88) | (5.13) |
| C12orf10 | 53911_at | (3.62) | (4.44) | (3.86) | (6.46) |
| CERKL | 60314_at | (4.68) | (3.03) | (7.37) | (3.62) |
| NOV | 39250_at | (3.20) | (3.90) | (4.38) | (7.37) |
| EPB41L5 | 60293_at | (4.33) | (4.97) | (3.06) | (3.92) |
| WNT5B | 66142_s_at | (3.94) | (3.87) | (4.49) | (4.16) |
| ACYP2 | 64090_s_at | (3.36) | (4.33) | (3.68) | (5.82) |
| C9orf103 | 56186_at | (3.14) | (4.62) | (4.03) | (3.73) |
| FBXO2 | 57811_at | (3.51) | (3.37) | (4.16) | (5.33) |
| CD38 | 40323_at | (3.25) | (3.37) | (4.27) | (4.27) |
| BCAS1 | 37821_at | (4.96) | (3.19) | (4.26) | (3.34) |
| TMSL8 | 36491_at | (3.03) | (4.11) | (3.45) | (7.67) |
| ISL1 | 39990_at | (3.12) | (3.78) | (3.61) | (3.91) |
| HSPB8 | 56474_at | (3.45) | (3.87) | (3.04) | (7.50) |
| B3GALT3 | 53879_at | (3.04) | (4.02) | (3.77) | (3.48) |
| CYBRD1 | 50955_at | (3.70) | (3.51) | (3.21) | (5.60) |
| EFEMP2 | 63644_at | (3.25) | (3.91) | (3.28) | (3.97) |
| TU3A | 45260_at | (3.14) | (3.94) | (3.22) | (4.82) |
| LOC57228 | 34176_at | (3.68) | (5.30) | (3.41) | (3.16) |
| IER2 | 36097_at | (4.79) | (3.20) | (3.11) | (3.88) |
| DKFZP564K1964 | 65860_at | (3.53) | (3.11) | (3.52) | (4.62) |

12.12 PATHWAY MODELS

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.

12.12.1 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:

$$\text{Akt}(k+1)=\text{NOT PTEN}(k)$$

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:

$$x_j(k+1) = (x_1(k) \text{ AND } x_3(k)) \text{ NOT } x_4(k) \text{ OR } x_6(k)$$

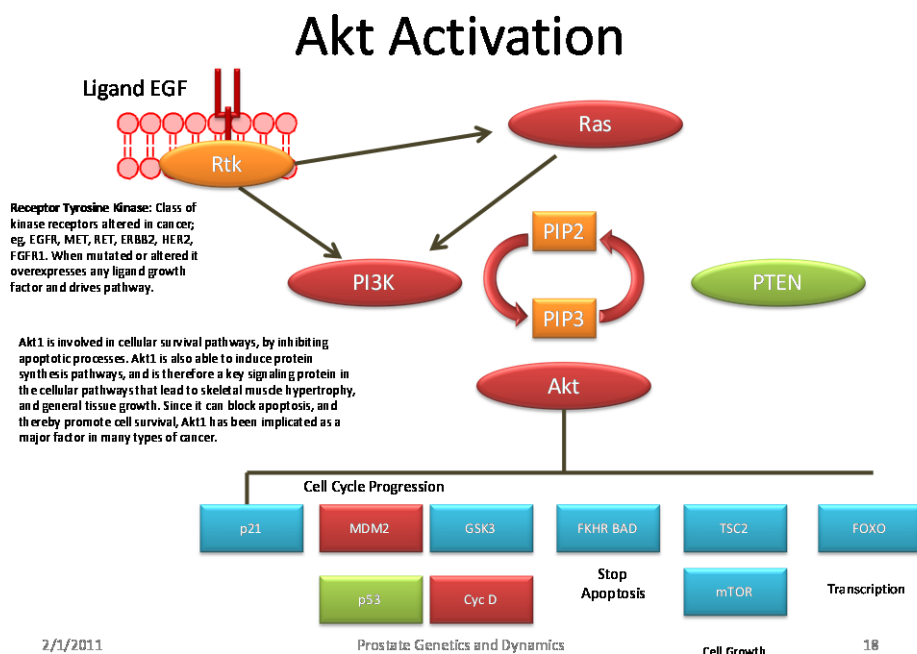
We now ask where did this equation come from. We defer that for the moment. But let us assume that we can find that for every one of the x values. Then we have:

$$x(k+1) = F(k+1, k)$$

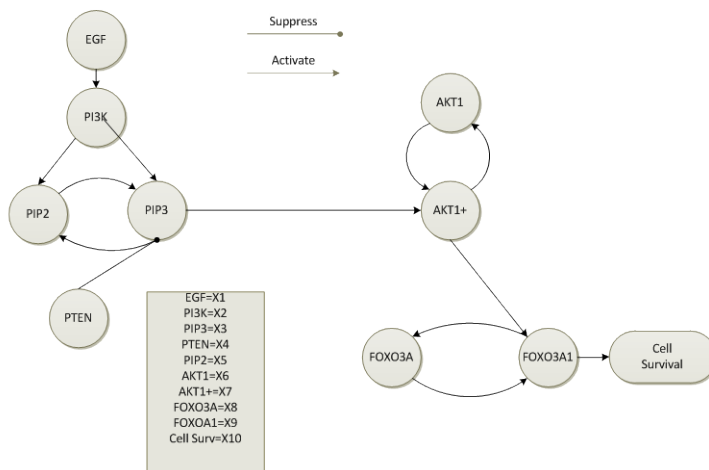
where

$$F(k+1, k) = \begin{bmatrix} f_1(k+1, k; x_1(k) \dots x_N(k)) \\ \dots \\ f_N(k+1, k; x_1(k) \dots x_N(k)) \end{bmatrix}$$

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;

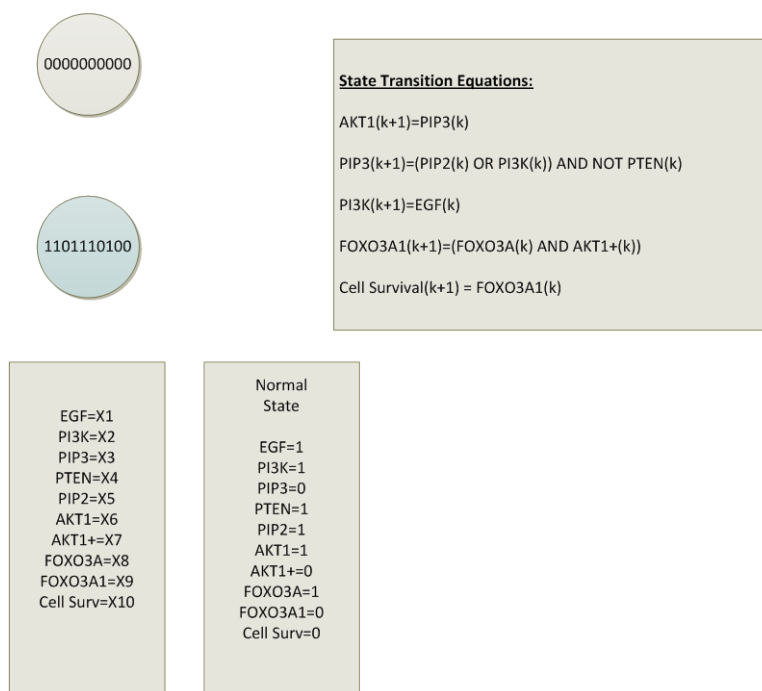


:
Then from the above we can generate a simple model of pathways with some detail as below:



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 work of Shmulevich et al (three papers in 2002, 2002, 2002)

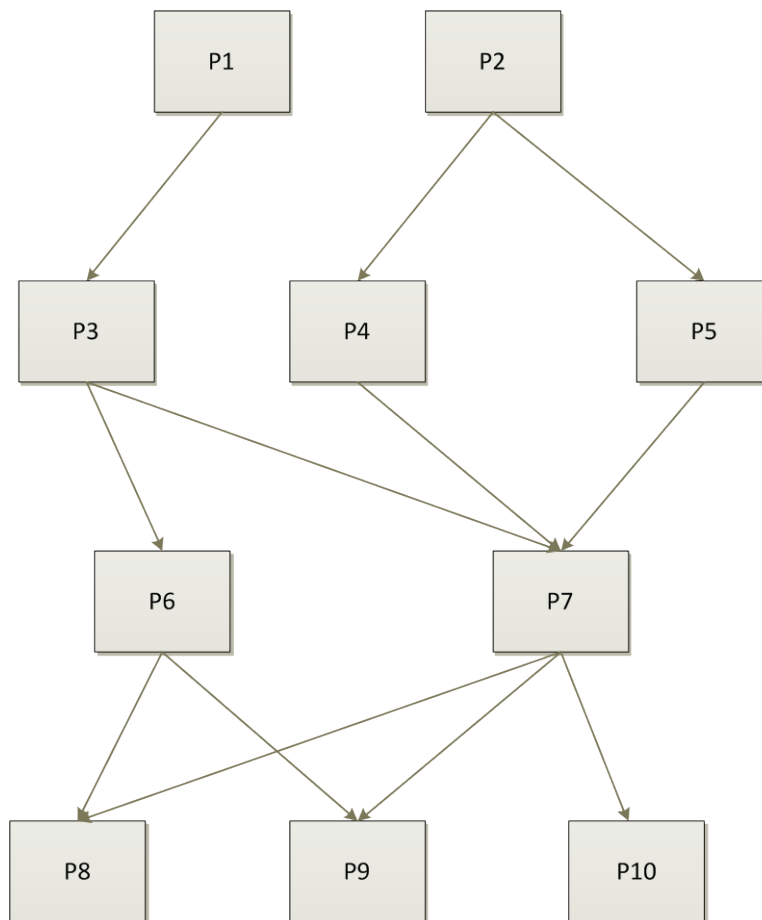
12.12.2 Bayesian Networks

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.

12.3.8 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):

$$p(P_{10}, P_9, P_8, \dots, P_1) = p(P_{10} | P_9, \dots, P_1) p(P_9 | P_8, \dots, P_1) \dots p(P_2 | P_1) p(P_1)$$

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 | Red | | Red | Red | | | | | | | Red | Red | Red | | | | | | Red | Red |
| P2 | Red | Red | | Red | Red | Red | Red | Red | Red | Red | | | | Red | Red | Red | Red | Red | | |
| P3 | Red | | Red | Red | | | | | | | | Red | Red | | | | | Red | Red | Red |
| P4 | Red | Red | | Red | Red | Red | Red | Red | Red | | | | | Red | Red | Red | Red | Red | | |
| P5 | | Red | | Red | Red | Red | Red | Red | Red | | | | | Red | Red | | Red | Red | | |
| P6 | Red | | Red | Red | | | | | | | | Red | | | | | | Red | Red | Red |
| P7 | | | | Red | | | | | | | | | | | | | Red | Red | | |
| P8 | | | | Red | | | | | | | | | | | | | Red | Red | | |
| P9 | | | | Red | | | | | | | | | | | | | Red | Red | | |
| P10 | | | | Red | | | | | | | | | | | | | Red | Red | | |

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:

$$\begin{aligned} \max \left[\log P[G|D] \right] = \\ \max \left[\log P[D|G] + \log P[G] + c \right] \end{aligned}$$

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:

$$\begin{aligned} P_i &= \sum_{k=1, \neq i}^{10} a_{i,k} P_k \\ \text{and} \\ d_i &= b_i P_i \end{aligned}$$

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:

$$\begin{aligned} P_i &= \sum_{k=1, \neq i}^{10} a_{i,k} P_k + n_i \\ \text{and} \\ d_i &= b_i P_i + w_i \end{aligned}$$

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):

$$\begin{aligned} \max \left[\log P[G|D] \right] = \\ \max \left[\log P[D|G] + \log P[G] + c \right] \\ \text{or} \\ \max \left[- \sum_{k=1}^{10} \sum_{i=1}^{20} \left(d_{i,k} - \hat{d}_{i,k} \right)^2 \right] + \log P[G] \end{aligned}$$

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-calculatable.

We demonstrate in the next section that we can measure analog values for the measurements as well as for the desired connections.

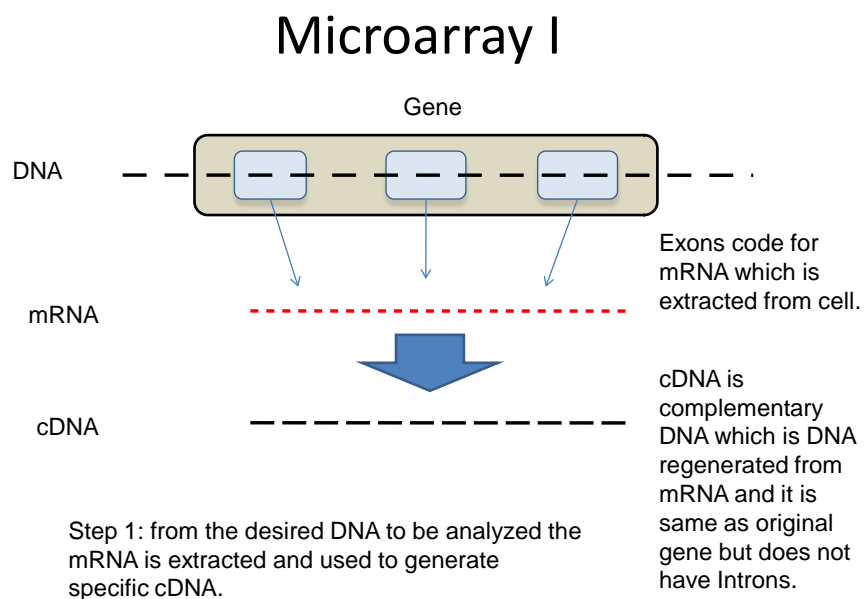
12.3.9 Microarrays

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.

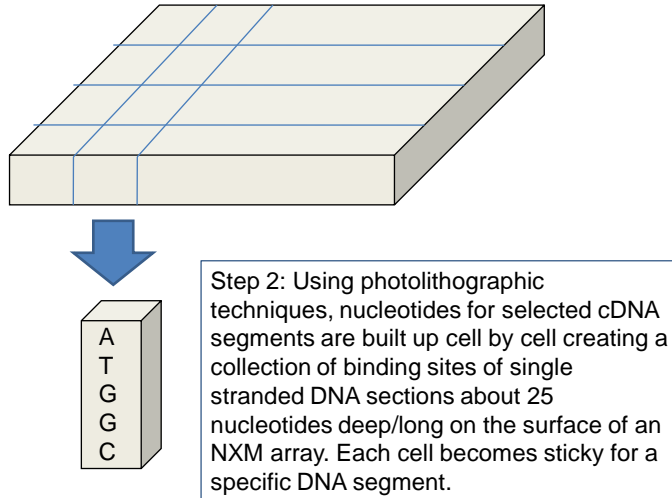
Step 1: The first step in a micro array is the production of cDNA, or complementary DNA. cDNA is that set of nucleotides which account for the encoding of mRNA. It does not include the non-coding regions which are the introns.



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

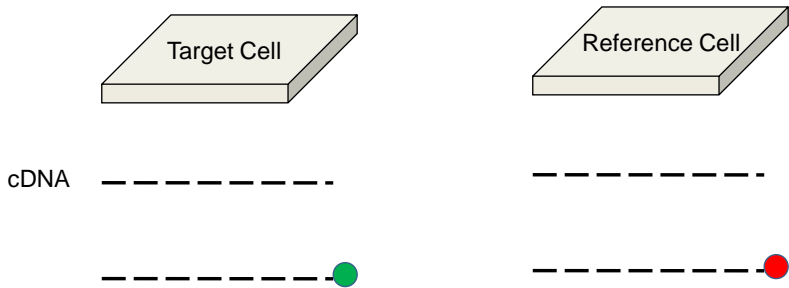
used nucleotides. The array has millions of small holes in an array like manner. Each hole we fill with nucleotide, one nucleotide at a time.

Microarray II



Step 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.

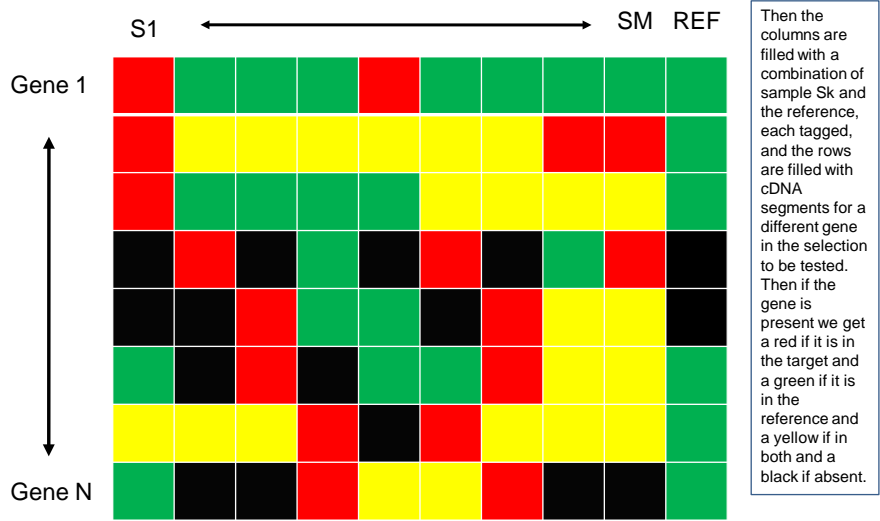
Microarray III



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.

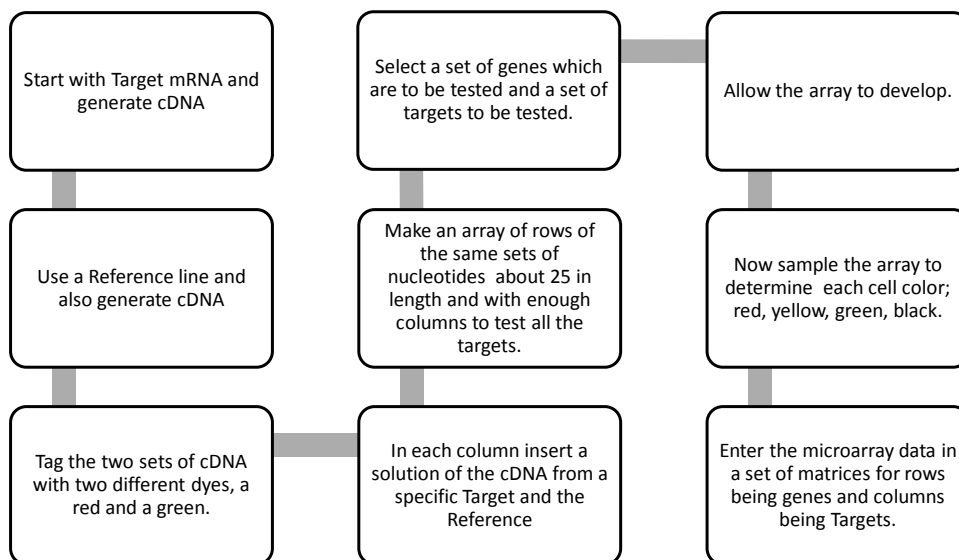
Microarray IV



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.

12.3.10 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.

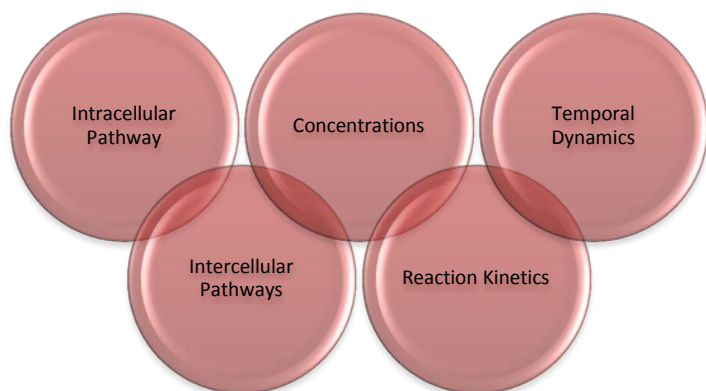
Thus Boolean and Bayesian are useful for identifying pathways whereas the full system model works when the pathway is somewhat well understood and we are looking more for the dynamics and details of the reactions. In a sense they are useful at different stages of the process.

12.12.3 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:

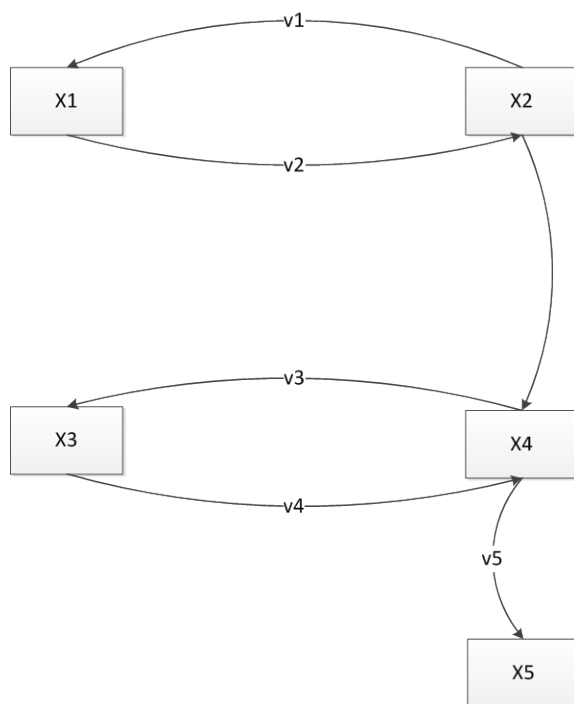
1. **Intracellular Pathways:** The description of pathways within the cell and a description which focuses on concentrations of products and exogenously extracellular products that affect the pathways.
2. **Intercellular Pathways:** This is the modeling of pathways between and amongst the cells. It is 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.
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:

$$\frac{d[x_1(t)]}{dt} = v_1 - v_2$$

$$\frac{d[x_2(t)]}{dt} = v_2 - v_1$$

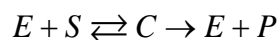
$$\frac{d[x_3(t)]}{dt} = v_4 - v_3$$

$$\frac{d[x_4(t)]}{dt} = v_3 - v_4$$

$$\frac{d[x_5(t)]}{dt} = v_5$$

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:



We further assume that the forward rate from E+S to C is k_1 and the reverse is k_{-1} and the forward rate from C to E+P is k_2 . Then we have the following:

$$\frac{dE}{dt} = -k_1ES + k_{-1}C + k_2C$$

$$\frac{dS}{dt} = -k_1ES + k_{-1}C$$

$$\frac{dC}{dt} = k_1ES - k_{-1}C - k_2C$$

$$\frac{dP}{dt} = k_2C$$

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:

$$\frac{dS}{dt} = -k_1(E_0 - CS) + k_{-1}C$$

$$\frac{dC}{dt} = k_1(E_0 - C)S - k_{-1}C$$

$$S(0) = S_0$$

$$C(0) = 0$$

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.

12.13 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 PCa. However, at this time we cannot differentiate these. Yet using the methodology as we develop it, we believe that understanding the dynamics of CSC PCa cells can be determined.

12.13.1 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.

12.13.1.1 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 known 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:

$$A = \begin{bmatrix} \frac{\partial f_1}{\partial x_1} & \dots & \frac{\partial f_1}{\partial x_n} \\ \dots & \dots & \dots \\ \frac{\partial f_n}{\partial x_1} & \dots & \frac{\partial f_n}{\partial x_n} \end{bmatrix}$$

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.

12.13.1.2 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. We may have samples from multiple cell conditions, that is we may have benign samples, HGPIN samples and PCa samples, and even divided by grade.

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 :

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

$$i = 1, M$$

Find

$$\hat{A} = \begin{bmatrix} \hat{a}_{1,1} \dots \hat{a}_{1,n} \\ \dots \dots \dots \\ \hat{a}_{n,1} \dots \hat{a}_{n,n} \end{bmatrix}$$

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:

$$g(a) = \sum_{k=1}^N (a_{11}x_1 + a_{12}x_2 + a_{13}x_3)^2$$

thus

$$\frac{\partial g}{\partial a_{11}} = \sum_{k=1}^N 2a_{11}x_1 (a_{11}x_1 + a_{12}x_2 + a_{13}x_3) = 0$$

or

$$\sum_{k=1}^N x_1 (a_{11}x_1 + a_{12}x_2 + a_{13}x_3) = 0$$

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.

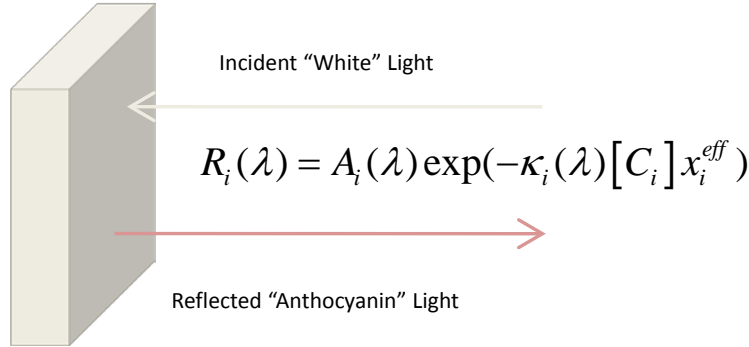
12.4 MEASUREMENTS

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 Law¹²⁰. 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.

¹²⁰ See Cantor and Schimmel, pp. 60-68.

Example



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41

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.

Here R is the reflected light we see at the wavelength specified and at the i th anthocyanin. A is the incident light amplitude at the wavelength specified. The exponent is Beer's law where C is the concentration of anthocyanin I and x the effective depth of that anthocyanin.

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.

$$R_{Total}(\lambda) = A(\lambda) \exp\left(-\sum_{i=1}^N \kappa_i(\lambda)[C_i]x_i(\lambda)\right)$$

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

$$\min \left(\sum_{m=1}^M \left(I(m) - \hat{I}(m) \right)^2 \right)$$

where

$I(m)$ is the m th 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.

12.5 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 coefficients¹²¹.
3. Steepest Descent: This is the incremental approach of best fit. It assumes we are trying to solve an optimization problem.

¹²¹ See Hildebrand pp. 478-494. The use of splines is an approach which tries to match coefficients of polynomials.

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.

12.5.1 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

$\tilde{R}(\lambda)$ 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 $[\hat{C}_i]$, $i=1\dots N$, such that

*$(\tilde{R}(\lambda) - \hat{R}(\lambda))^2$ is minimized where $\hat{R}(\lambda)$ is the
estimated received spectral element*

We may also characterize the variables as follows:

Let

$$x(k) = \begin{bmatrix} C_1 \\ \cdot \\ \cdot \\ C_n \end{bmatrix} = x(k+1)$$

and

$$z(k) = c^T(k)x(k) + n(k)$$

where

$$c(k) = \begin{bmatrix} -\kappa_1(k)x_1 \\ \cdot \\ \cdot \\ -\kappa_n(k)x_n \end{bmatrix}$$

and for this case k and λ are identical increments

We now consider three possible approaches.

12.5.2 Newton Steepest Descent

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:

$$\hat{a} = \begin{bmatrix} \hat{a}_1 \\ \vdots \\ \hat{a}_n \end{bmatrix} = \begin{bmatrix} [C_1] \\ \vdots \\ [C_N] \end{bmatrix}$$

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 Method¹²²:

¹²² 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:

$$\left[\frac{\partial g(a)}{\partial a} \right] = \begin{bmatrix} \frac{\partial g_1}{\partial a_1} & \dots & \frac{\partial g_1}{\partial a_n} \\ \frac{\partial g_n}{\partial a_1} & \dots & \frac{\partial g_n}{\partial a_n} \end{bmatrix}$$

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 $n \times 1$ 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.

12.5.3 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.

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

$$u(t) = \begin{bmatrix} u_1 \\ \dots \\ u_6 \end{bmatrix}$$

And we assume a system noise which is white with the following characteristic:

$$E[n(t)] = 0$$

and

$$E[n(t)n(s)] = N_0 I \delta(t - s)$$

Now we can define:

$$A = \begin{bmatrix} A_1 \dots 0 \\ 0 \dots A_2 \end{bmatrix}$$

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 anthocyanins are given by the 2 x 2 vector as follows:

$$\begin{bmatrix} z_1 \\ z_2 \end{bmatrix} = \begin{bmatrix} c_{11} \dots 0 \dots 0 \dots 0 \dots 0 \dots 0 \\ 0 \dots 0 \dots 0 \dots c_{24} \dots 0 \dots 0 \end{bmatrix} \begin{bmatrix} x_1 \\ x_2 \\ x_3 \\ x_4 \\ x_5 \\ x_6 \end{bmatrix} = Cx$$

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:

$$\frac{da(t)}{dt} = 0 : \text{where}$$

$$a(t) = \begin{bmatrix} a_1 \\ \dots \\ a_5 \end{bmatrix}$$

In this case we have assumed a is a 5×1 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 $m \times 1$ 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:

$$g(a, t) = g(a_0, t) + C(a_0, t)[a(t) - a_0(t)] +$$

$$\frac{1}{2} \sum_{i=1}^N \gamma_i [a - a_0]^T F_i [a - a_0] + \dots$$

Where we have:

$$C = \begin{bmatrix} \frac{\partial g_1}{\partial a_1} & \dots & \frac{\partial g_1}{\partial a_n} \\ \dots & \dots & \dots \\ \frac{\partial g_m}{\partial a_1} & \dots & \frac{\partial g_m}{\partial a_n} \end{bmatrix}$$

Thus we have for the measurement:

$$z(t) = C(t)a(t) + [g(a_0) - C(a_0)a_0(t)]$$

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

$$K\gamma(t-s) = E[w(t)w^T(s)]$$

In discrete time we have the equation:

$$\hat{a}(k+1) = \hat{a}(k) + PCK^{-1}[z(k) - \hat{z}(k)]$$

This is identical to the equation we derived from the Newton method.

12.5.4 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

$$\int_0^T s_1^2(t) dt = E_1$$

$$\int_0^T s_2^2(t) dt = E_2$$

and

$\exists \max$

$$\int_0^T s_1^{2,orth}(t) dt = \tilde{E}_1$$

$$\int_0^T s_2^{2,orth}(t) dt = \tilde{E}_2$$

Let us approach the solution using the theory of orthogonal functions¹²³. Now we can specifically use a Fourier series approach. We do the following:

¹²³ See Sansone, Orthogonal Functions.

Let

$$s_1(t) = \sum_{n=1}^{\infty} s_1^n \cos\left(\frac{2\pi}{T} nt\right) + r_1(t)$$

where

$$r_1(t) = s_1(t) - FS \cos$$

and

$$FS \cos = \sum_{n=1}^{\infty} s_1^n \cos\left(\frac{2\pi}{T} nt\right)$$

Likewise

Let

$$s_2(t) = \sum_{n=1}^{\infty} s_2^n \sin\left(\frac{2\pi}{T} nt\right) + r_2(t)$$

where

$$r_2(t) = s_2(t) - FS \sin$$

and

$$FS \sin = \sum_{n=1}^{\infty} s_2^n \sin\left(\frac{2\pi}{T} nt\right)$$

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"¹²⁴.

What we have sought to accomplish in this paper is to describe color and its 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 anthocyanins 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.

¹²⁴ See VanTrees, Detection, Estimation and Modulation Theory. He presents details on this solution.

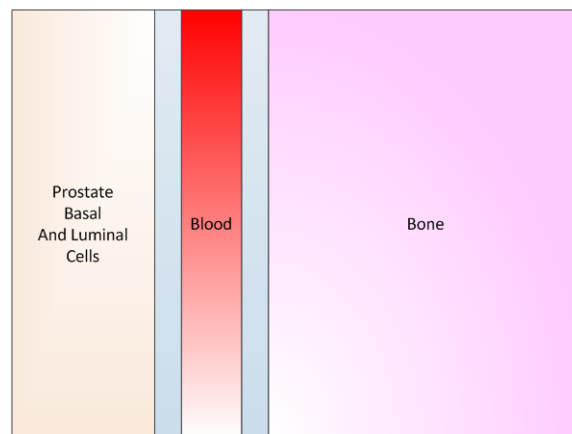
13 TOTAL CELLULAR DYNAMICS

Total Cellular Dynamics considers the problem of the movement of cancerous cells across the human body driven by both the internal genetic changes in the cell itself and the drivers from the external environment, the external environment of the benign cells and the communications from malignant cells.

In this chapter we introduce a simple model of such a system. Let us begin with a simple example. In this case we will examine the number of cancer cells as a function of time and as a function of location. Let us consider the following simple diagram. It consists of:

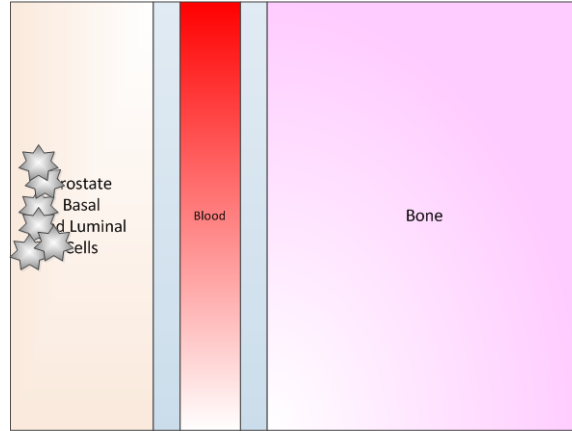
1. Prostate
2. Capillary Walls
3. Blood
4. Bone

We show it graphically below:



Now we assume the following:

1. A basal or luminal cell in the prostate undergoes a malignant mutation resulting in a localized growth. Thus we have a collection of malignant prostate cells growing at a single site as we show below. This is called time dependent local growth. The malignant prostate cells do not really go anywhere they just begin to bunch up. This is the growth phase. Namely at some point x at some time t the number of malignant prostate cells are $n(x,t)$ and growing.

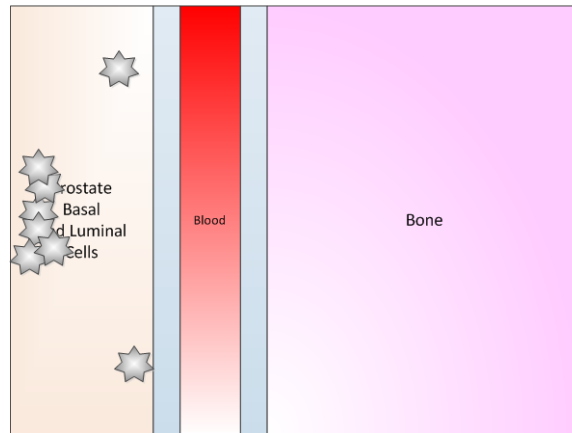


Thus in this phase we have:

$$\frac{\partial n(x,t)}{\partial t} = \gamma n(x,t)$$

That is the number of malignant cells are just expanding. But the expansion is local.

2. Now we start to see some movement. This movement is diffusional in nature, namely the malignant prostate cells begin to move from a high density out to lower density locations. The driver for increased malignant prostate cells in the first step was one set of cellular mutations but now when we start the diffusion we are driven by a second set of malignant mutations. The malignant prostate cells are driven by this cell change.

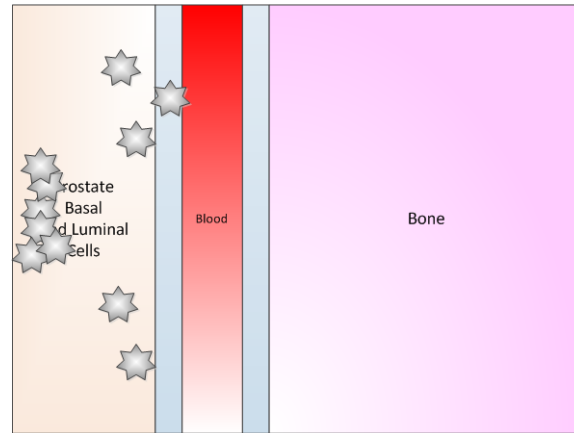


In this phase the movement is driven most likely by some diffusion, while growth also continues. Here we get:

$$\frac{\partial n(x,t)}{\partial t} = \alpha \frac{\partial^2 n(x,t)}{\partial x^2} + \gamma n(x,t)$$

That is we have growth at sites where a malignant cell is and we have a diffusion of the malignant cells. Before we had no diffusion, we had localized malignant cells. We wonder what cause the activation of the diffusion. We also have assumed, without any real basis that it is not flow, but only diffusion.

3. Now malignant prostate cells approach the capillary walls, the barrier to the blood system. What makes them cross that barrier? It is most likely another strong diffusion factor or perhaps a flow factor. This appears to be unknown at this time. But they do cross the capillary wall. We show this below.

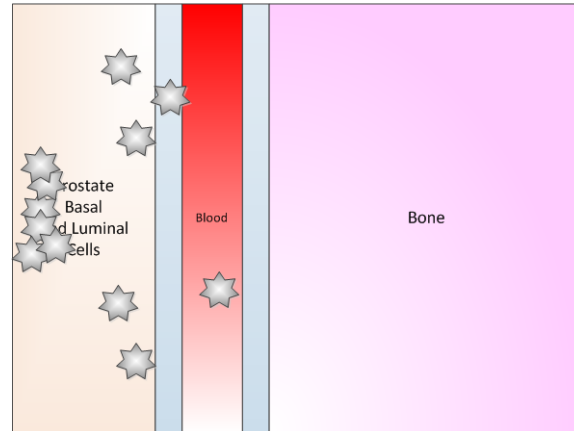


We also assume that there is no growth stimulants in the capillary wall and that further there is always just diffusion. We also assume no flow, that the capillary walls are flow free and growth free regions, that there is some diffusive flow only. This means that here we have:

$$\frac{\partial n(x,t)}{\partial t} = \alpha \frac{\partial^2 n(x,t)}{\partial x^2}$$

As the controlling description of the status of number of malignant cells.

4. Now the malignant prostate cell enters the blood stream. The movement in the blood stream is purely flow related. It just flows around until it is captured by another capillary wall. There is no growth in the blood stream, there are few growth factor ligands, but the capture at a capillary wall is still not understood.

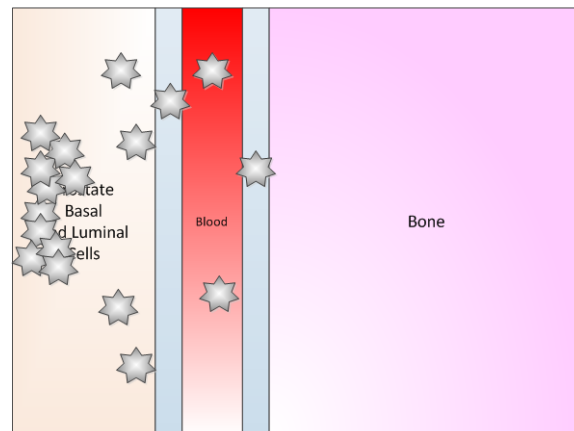


Now in the blood stream we assume that it is devoid of any growth factors and also that the flow of the blood is such that diffusion will not occur so that here we have only:

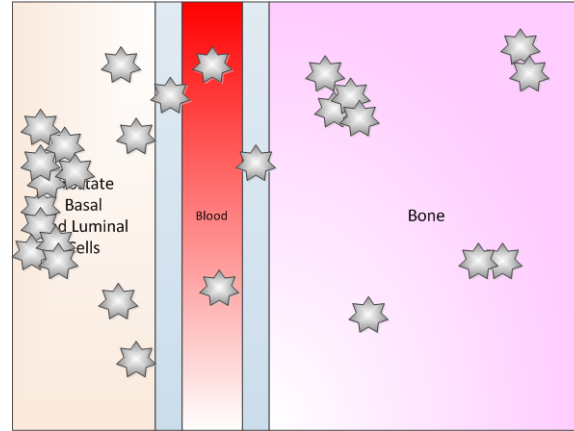
$$\frac{\partial n(x,t)}{\partial t} = \beta \frac{\partial n(x,t)}{\partial x}$$

This is a pure flow model.

5. We now show wall capture and movement through the other wall. This is the point at which the malignant prostate cell selects, if you will, a drop off point, and targets a specific organ. Perhaps it is some diffusive pull on the cell from some ligands in the targeted organ.



6. Finally the malignant prostate cells cross the capillary wall via some diffusive process and ends up in the bone where again it sees growth factors and also diffusive factors and then this is where metastasis begins.



In the bone or the metastatic site we have diffusion and growth again. Namely we have:

$$\frac{\partial n(x,t)}{\partial t} = \alpha \frac{\partial^2 n(x,t)}{\partial x^2} + \gamma n(x,t)$$

These simple steps describe the model which we will develop herein. It is a growth, flow, diffusion model where we focus on the number of malignant cells at specific times and locations.

13.1 ASSUMPTIONS

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.

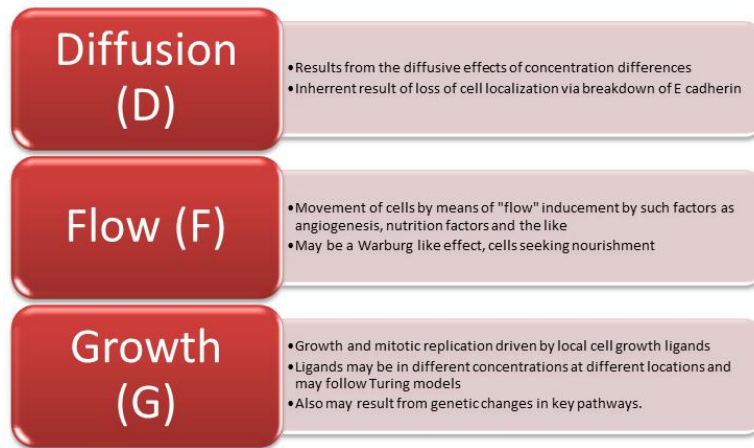
13.2 TOTAL CELLULAR DYNAMICS MODELS

Now depending on the case we assume we can model the flow of cancer cells according to some simple dynamic distributed models¹²⁵. Thus we could have for some measure of malignant cells $p(x,t)$ the following:

$$\frac{\partial p(x,t)}{\partial t} = D(x,t) \frac{\partial^2 p(x,t)}{\partial x^2} + E(x,t) \frac{\partial p(x,t)}{\partial x} + F(x,t) p(x,t)$$

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. Note also that we can readily make this a three dimensional model as well.

Now this description has certain physical realities.

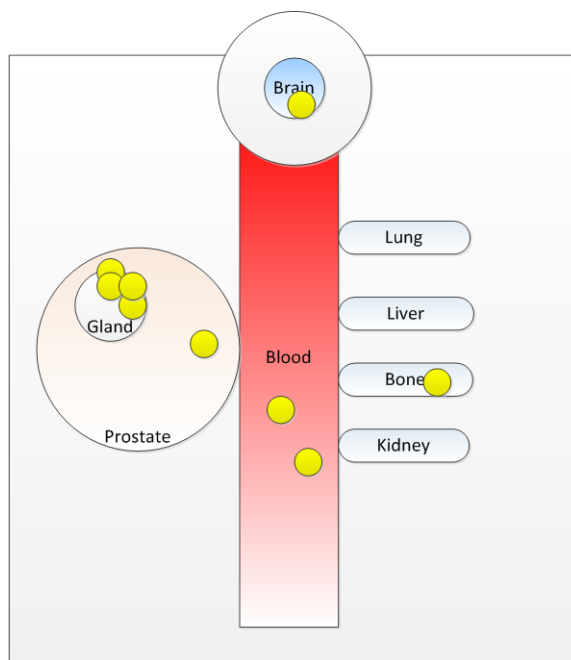


Here above we describe the three factors in terms of their effects and their causes. The three elements of the equation; diffusion, flow, and growth, are the three ways in which cancer cells move. We can summarize these as below:

¹²⁵ 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.

| 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 malignant prostate cells 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. |

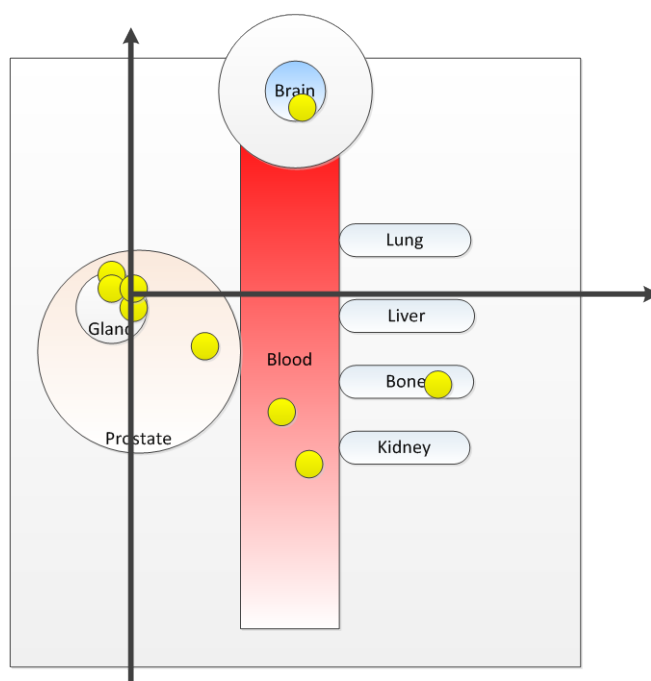
Now consider the following graphic as a human body,



We then divide the body up into different sections and then we assume each is uniform within itself. We have not included the capillary walls just for simplicity. We have a D, E, F, for each gross portion of the body. We also have a model as specifically below in the Table:

| <i>Organ</i> | <i>D Diffusion</i> | <i>E Flow</i> | <i>F Production</i> |
|------------------|------------------------|-------------------|-------------------------|
| 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.

13.2.1 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.

13.2.2 Determining the Coefficients

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.

13.2.3 Implications

There are many implications of this model. We consider here the possible implications when one looks at non-cancerous cells becoming effectors of cancerous cells in a distributed manner. This seems to be the results of recent efforts as published.

There is discussion by Bridger about how researchers now believe the environment, micro environment, can be a controller to cancer cell¹²⁶.

They state:

The research team has found that normal cells that reside within the tumor, part of the tumor microenvironment, may supply factors that help cancer cells grow and survive despite the presence of anti-cancer drugs. These findings appear online this week in a paper published in Nature.

"Historically, researchers would go to great lengths to pluck out tumor cells from a sample and discard the rest of the tissue," said senior author Todd Golub director of the Broad's Cancer Program and Charles A. Dana Investigator in Human Cancer Genetics at the Dana-Farber Cancer Institute. Golub is also a professor at Harvard Medical School and an investigator at

¹²⁶ http://www.eurekalert.org/pub_releases/2012-07/biom-tmh070212.php

Howard Hughes Medical Institute. "But what we're finding now is that those non-tumor cells that make up the microenvironment may be an important source of drug resistance."

We have argued likewise in a paper we wrote in early 2012. There we argued that such cancers as melanoma have a compelling model for metastasis which uses both short distance micro environment control as well as long distance macro environment signalling.

Namely we have modeled melanoma metastasis as a quasi-distinct organism using the human as a host and specifically using the host extracellular signalling as a means for allowing the stem cell to affect metastasis at a distance.

In the [Nature](#) article by Straussman et al¹²⁷, they state (also see write up by [Carpenter](#)¹²⁸):

Drug resistance presents a challenge to the treatment of cancer patients. Many studies have focused on cell-autonomous mechanisms of drug resistance. By contrast, we proposed that the tumour micro-environment confers innate resistance to therapy.

Carpenter states:

The presence of these cancer-assisting proteins in the stromal tissue that surrounds solid tumours could help to explain why targeted drug therapies rapidly lose their potency.

Targeted cancer therapies are a class of drugs tailored to a cancer's genetic make-up. They work by identifying mutations that accelerate the growth of cancer cells and selectively blocking copies of the mutated proteins. Although such treatments avoid the side effects associated with conventional chemotherapy, their effectiveness tends to be short-lived. For example, patients treated with the recently approved drug vemurafenib initially show dramatic recovery from advanced melanoma, but in most cases the cancer returns within a few months.

The Carpenter article concludes:

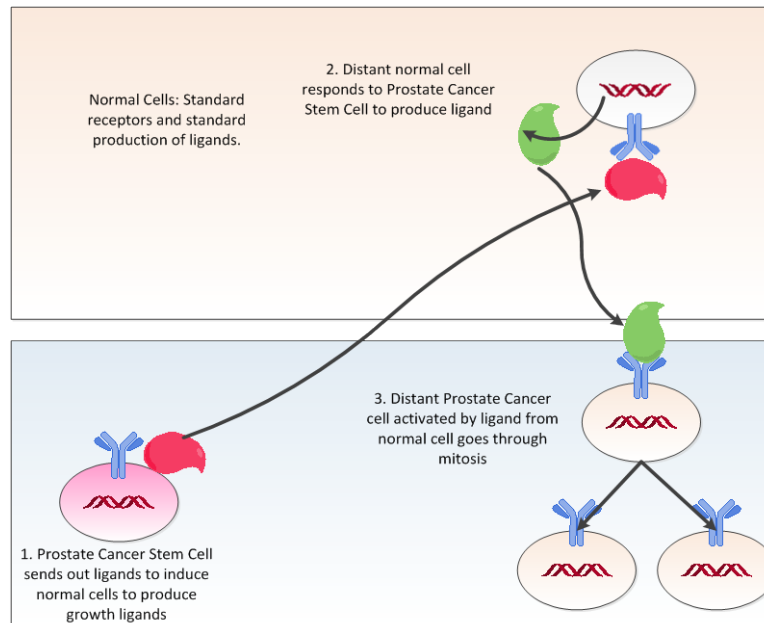
One of the most startling results of the teams' experiments was the discovery that a protein called hepatocyte growth factor (HGF) boosts melanoma's resistance to treatment with vemurafenib. Intrigued by this result, both teams looked at blood samples from people who had undergone treatment with vemurafenib, and found the higher a patient's HGF levels, the less likely they were to remain in remission.

We propose an alternative but what we believe to be a consistent interpretation. Consider the example below. We have conjectured based upon modeling that cancer may act as a separate entity on the human host and further that it uses the human host not only for nutrients but for communications. In fact using the results from this paper one can construct a verifiable model of

¹²⁷ Straussman et al, Tumour micro-environment elicits innate resistance to RAF inhibitors through HGF secretion, Nature, Published online 4 July 2012, <http://www.nature.com/nature/journal/vaop/ncurrent/full/nature11183.html>

¹²⁸ <http://www.nature.com/news/neighbouring-cells-help-cancers-dodge-drugs-1.10952>

a bi-system distributed environment. Here the prostate cancer cell uses a stem cell to communicate at a distance.



The above is a hypothetical example:

1. There exists a prostate cancer stem cell. It can produce ligands which manage to use the body's distribution system; blood or lymphatic.
2. The ligands use normal health cells which are to be activated and in turn produce at a distant site growth ligands at that site.
3. At the distant site we have prostate cancer non stem cells which respond to this massive influx, an amplifier system if you will, to make the non-stem melanoma cells to proliferate.

This is just an interesting but possible physical interpretation.

13.3 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.

13.4 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.

13.5 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

13.6 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.

This may sound a bit arcane but it does have a function here. Remember that we have a system where the state is the number of malignant cells at any point and at any time. We know that the number is determined by the complex set of equations we have developed but that the set of equations is “driven” by the expression of certain proteins expressed by not only the malignant cell but also by the benign environment the malignant cells find themselves in.

Thus we may pose the following question:

“How can we drive the $n(x,t)$ at some distant points, such as the bone or liver, to zero by adjusting the cells driver coefficients?”

A corollary to this is the question where we say:

“What cells do we activate or suppress in the normal tissues, or what ligands do we add to act as such, so as to drive the $n(x,t)$ to zero in the metastasized locations?”

The controllable aspects of the system we have developed can be then used to conceive of potential approaches to control the disease.

13.7 OBSERVABILITY

A system is observable if from a set of measurements, $z(t)$, where $t \in (r,s)$, 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.

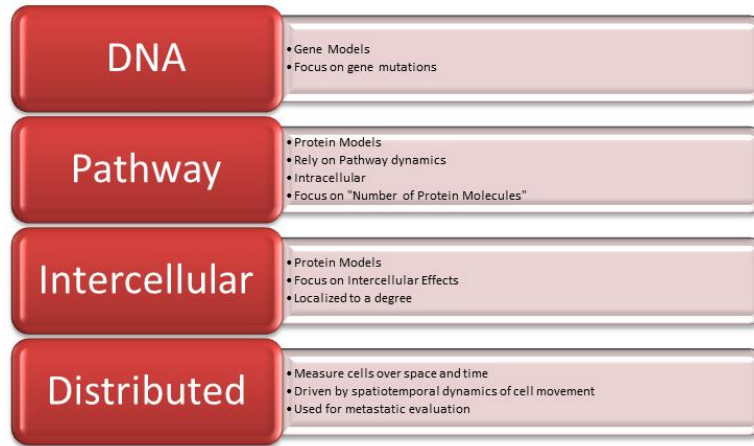
Thus with these two concepts we have a model for a system which can be used to both identify all states and drive it to a desired state.

13.8 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 an 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.

The model developed in this Chapter is one to enable the researcher to view cancer as a system disease, yet one which has some semblance of control. The knowledge of what activates pathways and in turn the flow of cancerous cells is also a window on how to control them.

14 CONCLUSION

We have completed our model, analysis and development tools regarding PCa. As any researcher knows, the moment we take “pen from paper”, there will be many new developments, each of which will either add to what we have already said, or may even change our current mode of thinking. As we had indicated at the commencement of this effort, our intent was not to provide the sine qua non document of pathways and PCa but to present a way of thinking of the problem in a more expansive yet holistic manner. Namely we want to view PCa as something which on the one hand may be a single cell directed yet on the other may be quite systems expressive in a fully distributed manner.

14.1 PREAMBLE

In this conclusion section we do not summarize what we have presented, we allow the document to speak for itself. What we do is lay out several other areas of discussion which tend to continue the back and forth that one sees in PCa research. Let us summarize them here briefly:

1. HGPIN and is it a precursor? This issue has been a driving factor in many investigations in the research of PCa. As Goldstein and others have argued there is an unstoppable progression from benign cells to PCa and it moves through HGPIN. It is almost easy to say from the work of the researchers that it is inevitable for PCa to evolve from HGPIN. However as we have anecdotally seen, there are a limited number of cases of HGPIN becoming benign. The question is why. The answer may hold considerable insight to understanding PCa.

2. PSA and the USPTF: This discussion brings us back to the political and real world elements associated with PCa. The USPTF is fundamentally a political organization composed of a mix of individuals chose less for their disease specific competence and as it appears more for their political connections. The net result is that when the USPTF opines on some topic it has far reaching influence despite the fact that they rely to a very limited extent on those in the data to day fight against the disease, as is the case with PCa. Thus unlike so many other diseases, PCa has advocates and detractors, and currently the Government and its funding arms are on the detractor side.

3. PSA Effectiveness and Proper Tests: We also examine some of the more recent results postdating the USPTF. As we have done with prior results we again demonstrate that the wrong question was asked. The faults are often clearly evident, as with the case we discuss, namely too long a period for testing. We have argued that PSA has value but value if and only if it is used properly.

4. Metabolic Factors: The classic Warburg hypothesis which focuses on the changes from aerobic to anaerobic growth of cancer cells, and Warburg’s famous experiments related thereto, have from time to time sparked interests in this area. While we find it of interest it does not seem to fit readily in our analysis.

5. Immune System and PCa. We have discussed some of the immune system markers in our chapter on Prognostic Factors but unlike other cancers there seems to be a more limited amount

of research regarding this for PCa. We do not again discuss the immune issues but we believe that we will discover as we have in such disorders as Type 2 Diabetes, that the inflammatory processes and immune system balance will play a significant modulating role.

14.2 HGPIN AND A PRECURSOR

We have examined HGPIN and the genetic defects observed in PCa. The answers to progression from benign to HGPIN to PCa are not at all well understood. There is a correlative relationship between gene loss or enhancement and the stage of PCa but there clearly is no definable and replicable path. PTEN is lost in some but not all, and the same holds for all of the genes and their aberrations. There is not a common and predictable pathway at this time. The pathway to understanding the forward progression is filled with many paths. Perhaps that is just the very nature of this cancer. It lacks the consistency of a Vogelstein model that colon cancer has, but in many ways it is a much more typical cancer.

One of the most obvious conclusions is that an effective model of prostate genetic dynamics is still a work in progress. There are several factors which limit what we can accomplish:

1. The details regarding the effects of the genes that have been targeted are still qualitative and not adequately detailed.
2. The complete epigenetic networks are not fully complete. For example with regard to PTEN or the AR genes, we do not know if they are eliminated or epigenetically suppressed and if the later by what specific mechanism, methylation, miRNA, or other gene products yet to be determined.
3. The mechanism for regression is not well understood. The presence of p27 or GSTP1 and other genes is suggestive at best and not necessarily causal. It will be necessary in this case to work through full details. In addition, there is the issue of immune response and ligand control.

We have focused on pathways and the dynamics of the reaction kinetics. The primary focus has been within cells. There are reasons for understanding the detailed concentration dynamics. As we have seen, many researchers have developed models which are understandings of pathways at the highest levels, namely what products relate to what other products. Other researchers as they delve into the dynamics of the pathways have employed the Boolean and Bayesian methods. We have argued that these methods are tuned for ascertaining pathway structure and not necessarily pathway dynamics. We have argued that detailed dynamic concentration models must be used.

To understand why the concentrations and their dynamics are useful we look at a recent paper by Carracedo et al where they are discussing PTEN and the authors state:

The importance of PTEN (phosphatase and tensin homolog located on chromosome 10) in cancer has surpassed all predictions and expectations from the time it was discovered and has qualified this gene as one of the most commonly mutated and deleted tumor suppressors in human cancer. PTEN levels are frequently found downregulated in cancer, even in the absence

of genetic loss or mutation. PTEN is heavily regulated by transcription factors, microRNAs, competitive endogenous RNAs (such as the PTEN pseudogene), and methylation, whereas the tumor suppressive activity of the PTEN protein can be altered at multiple levels through aberrant phosphorylation, ubiquitination, and acetylation. These regulatory cues are presumed to play a key role in tumorigenesis through the alteration of the appropriate levels, localization, and activity of PTEN. The identification of all these levels of PTEN regulation raises, in turn, a key corollary question: How low should PTEN level(s) or activity drop in order to confer cancer susceptibility at the organismal level? Our laboratory and others have approached this question through the genetic manipulation of Pten in the mouse. This work has highlighted the exquisite and tissue-specific sensitivity to subtle reductions in Pten levels toward tumor initiation and progression with important implications for cancer prevention and therapy.

The very title of their paper, namely looking at what amount of PTEN is too little frames the question well.

Now looking at a single cell is but one step. We also believe that one must look at the inter-cell signalling as well. Thus the spatial dynamics of inter-cell signalling we believe will also play a significant role in cancer development. We have examined the issue of inter-cell communications when looking at the control of secondary pathways elsewhere. However in the development of cancer and its propagation we have little to begin with other than recognizing its importance.

Having described the multiple genes and their products who presence or absence is identified with PCa and having further identified the pathways and then their dynamics in terms of measurable results, we now seek to ask two additions questions.

First, how can we identify the many constants in the models which we have developed? Namely can we observe the model in adequate detail so as to have confidence in what has been speculated?

Second, if we can observe the models to a reasonable degree of accuracy and predictability, can we then develop means and methods to control the actual system, and be capable of driving it from one state to another? Can we control PCa or even more so can we regress PCa?

Apoptosis is the normal progression and death of cells for a variety of reasons and predominantly because they are no longer functional, no longer do what they should be doing. Apoptosis is self-recognized in the cell and the cell takes it upon itself to die. The loss of this capability is one of the major factors in the development of a cancer. The existence of this factor in HGPIN remission may be a natural path to returning to normality. But the question still remains; what causes the remission, and if it is apoptotic then what makes it so?

The immune system responds when it perceives some antigen, a non-self-indicator on a cell, which initiates a set of cascades within the complexity of the immune system to recognize the invader and take actions to cleanse the body of this invader.

14.3 SCREENING FOR PROSTATE CANCER: THE TASK FORCE REPORT

The U.S. Preventive Services Task Force has issued its report regarding screening for prostate cancer¹²⁹. We will make a few observations here based upon what we have developed herein and other recent efforts. Let us begin by saying that not all prostate cancer is the same and at this time no one really knows how to determine the difference. This is a genetic pathway problem and the issue is that many elements of the pathway are yet to be identified and moreover the dynamics of the pathway are still unclear.

- 1. Does screening for prostate cancer with PSA, as a single-threshold test or as a function of multiple tests over time, decrease morbidity or mortality?*
- 2. What are the magnitude and nature of harms associated with prostate cancer screening, other than overtreatment?*
- 3. What is the natural history of PSA-detected, nonpalpable, localized prostate cancer?*

Let us examine these questions based upon the Task Force's approach and based upon our analyses and compare them.

1. DOES SCREENING FOR PROSTATE CANCER WITH PSA, AS A SINGLE-THRESHOLD TEST OR AS A FUNCTION OF MULTIPLE TESTS OVER TIME, DECREASE MORBIDITY OR MORTALITY?

Task Force:

No good- or fair-quality RCTs addressed this question. Two poor-quality RCTs with important flaws in design and analysis do not show a mortality benefit from PSA screening independently or in a meta-analysis. We identified no RCTs that measured health outcomes from PSA screening by means other than single-threshold tests.

Now would one be willing to bet one's life on 2 poor tests! Also as we have noted elsewhere the tests reported in NEJM a year or so ago were flawed for several reasons, mainly they used a fixed and out dated threshold, and in fact asked the wrong question, and also especially in the European study tests at too high a level and tested too infrequently.

The medical issue is that the PCa which is the most deadly is also the fastest growing and it should be tested at a lower level and more frequently. The remaining PCa is really indolent, no one denies that, but the determination of which is which is difficult without sophisticated genetic tests.

2. WHAT IS THE MAGNITUDE AND NATURE OF HARMS ASSOCIATED WITH PROSTATE CANCER SCREENING, OTHER THAN OVERTREATMENT?

¹²⁹ <http://www.uspreventiveservicestaskforce.org/prostatecancerscreening.htm>

Task Force:

One cross-sectional and 2 prospective cohort studies of fair-to-good quality reported short- and long-term psychological harms from prostate cancer screening. Although abnormal screening results did not affect summary measures of anxiety or health-related quality of life, men with false-positive PSA screening test results were more likely to worry specifically about prostate cancer, have a higher perceived risk for prostate cancer, and report problems with sexual function compared with control participants for up to 1 year after the test. In 1 study, 26% of men with false-positive screening results reported moderate- to-severe pain during the prostate biopsy; men with false-positive results were also more likely to undergo repeated PSA testing and additional biopsies.

The counter to this is simple, many people are just terrified about death, yet it comes to all. Are there issues for some men, yes, but those most likely are the same men who smoke, drink in excess, are obese, and the list goes on. Is a prostate biopsy painful, it is a state of mind and medication?

This second issue seems to be a contrived issue. People have the same issue regarding colonoscopies, which have been clinically effective in reducing death from colon cancer. Yet we seem not to hear this issue. The Task force also had the same concern about mammographies.

Frankly why should this be an issue for the Task Force at all. The public will talk but let an educate patient decide, it is ultimately the patients choice.

3. WHAT IS THE NATURAL HISTORY OF PSA-DETECTED, NONPALPABLE, LOCALIZED PROSTATE CANCER?

Task Force:

Three fair-quality cohort studies with small-to-medium sample sizes, highly self-selected elderly patients, and high drop-out rates show that some men with PSA detected, nonpalpable, localized (stage T1c) prostate cancer have good health outcomes up to 10 years after diagnosis. We did not identify any population-based studies in which patients with stage T1c prostate cancer were followed longitudinally with no intervention in order to determine health outcomes resulting from the natural progression of disease.

It appears that the Task Force has no answer here. In fact they asked the wrong question. They should have asked what genetic markers were prognostic of a virulent form of PCa. Simple question, but we as of yet really do not know. Assume we knew? Then What? Can we test every cell for these genes? What of the issue of a cancer stem cell, thus there being say just a few hundred or ten of them, and must we find them? Do they give off a measurable inter-cellular marker to express their presence?

The answer seems to be we do not yet know. How will we find out? More studies with more men. Yes more biopsies where the samples are analyzed genetically in a large scale study. Yet with the admonitions given off by the Task Force that may soon become unlikely.

A recent study on the genetic level in Oncology states¹³⁰:

Arul M. Chinnaiyan and colleagues (sic) sought to develop a new read-out for prostate cancer due to the non-specificity and unclear mortality benefit of PSA testing. The goal was to find a novel biomarker or biomarkers that can facilitate the individualization of PSA levels.

The test the researchers developed is a clinical-grade, transcription-mediated amplification assay that can detect prostate cancer non-invasively in the urine. The read-out of the test is the quantitative measure of a TMPRSS2: ERG fusion transcript that is unique to prostate cancer. More than 50% of PSA-screen prostate cancer harbors this fusion between the transmembrane protease, serine 2 (TMPRSS2) and the v-ets erythroblastosis virus E26 oncogene homolog (avian) (EGR) gene.

Thus we are beginning to see such tests arising. Yet, there is not a comment from the Task Force regarding this work. This is the essence of the Translational approach, the translating of science into the practice of medicine. This is another shortfall of the Task Force.

Now let us return and review what the Task Force said. The Task Force total basis of their conclusion seems to be as follows:

Effectiveness of Early Detection and Treatment: *A meta-analysis of **2 poor-quality** RCTs (random controlled trials) of population- based screening for prostate cancer using PSA and digital rectal examination found no reduction in prostate cancer mortality in men invited versus men not invited for screening (relative risk, 1.01 [95% CI, 0.80 to 1.29]). A recent RCT reported that men who received PSA screening had a decreased risk for receiving a diagnosis of metastatic prostate cancer. The USPSTF assessed the study as providing inconclusive evidence of benefit from screening because of a high likelihood of unequal outcome ascertainment and small absolute numbers of an imperfect intermediate health outcome (metastatic prostate cancer is an imperfect surrogate of prostate cancer mortality because of both high initial response rates to androgen deprivation therapy and competing causes of death). No RCTs have reported health outcomes from the variations of PSA screening that consist of multiple measurements over time (for example, measurements of PSA velocity, PSA slope, or PSA doubling time).*

However that is the point. We examined many of these trials and we concluded:

1. PSA thresholds must be age dependent as well a family history dependent.
2. PSA velocity is an essential element of the analysis and it means that PSA tests should be performed annually starting say at 30 to 35. Thus PSA velocity can be reasonably determined.
3. PSA testing needs a better baseline as a test. There is substantial variation between PSA values based on different testing methods.
4. PSA measurements including % Free PSA are also useful and should equally be used.

¹³⁰ <http://www.cancernetwork.com/prostate-cancer/content/article/10165/1922881>

5. PSA algorithms can assist in ascertaining what may be a cancer but a biopsy is usually required.

6. Biopsies

One of the conclusions is as follows:

***How Does Evidence Fit with Biological Understanding?** Prostate-specific antigen screening presupposes that most asymptomatic prostate cancer cases will ultimately become symptomatic cases that lead to poor health outcomes. However, the natural history of PSA-detected, nonpalpable, localized prostate cancer is poorly described. No prospective studies have followed a population-based cohort of patients with screening-detected cancer who have had no intervention in order to determine health outcomes resulting from natural progression of the disease. Evidence from small, selected cohorts of men with arbitrarily defined “favorable risk” (that is, with prostate cancer likely to be clinically indolent) suggest a good prognosis for some men with screening-detected cancer; however, the longest of these studies has reported health outcomes from 2 to 10 years after diagnosis only.*

The statement is evidential hearsay at best. The problem is that prostate cancer, like so many cancers, is still somewhat of an enigma, but it is fair to say that it is a genetic pathway breakdown and this breakdown results in expanding cells that lose the ability to understand where they belong, thus metastasis. The Report totally fails to join this issue. The above is about as close as it appears to get.

Now the Press as usual is in the fray. The NY Times reports¹³¹:

But doctors are divided about when to recommend watchful waiting. The decision can be guided by an indicator called the Gleason score, a measure of the aggressiveness of the cancer found in a biopsy, but there is often disagreement about how to care for men whose scores are in the middle — neither highly aggressive nor probably not aggressive. In addition, the biopsy process itself is imprecise; a standard “12-core biopsy” gives information about only one three-thousandth of the prostate, says Dr. Eric Klein of the Cleveland Clinic. According to research at Johns Hopkins, staging and grading mistakes occur in about 20 percent of specimens.

The Gleason score is a microscopic pathological marker. Simply put are the prostate glands normal, small opening with evenly distributed basal cells with luminal cells atop? Or are the glands reproducing, many small immature glands, then expansion of half formed glands, and then just a mass of uncontrolled cells, moving from Gleason 1 through 5 and Gleason 2 through 10 on its score. That may be useful but it totally fails to tell us the genetic makeup of the cancer stem cell controlling this entire process.

¹³¹ <http://well.blogs.nytimes.com/2011/10/10/prostate-test-finding-leaves-a-swirl-of-confusion/?nl=health&emc=healthupdateema2>

The goal is determining the genetic make-up, that is, what should be the topic, not this foolishness.

As a final conclusion: one should look at the number of studies used, 3 for the first question, three for the second, and 3 for the third. Yes they scanned a thousand or so, but only 9 studies were used. We believe that this is too limited.

Welch, one of the authors of "Overdiagnosed" wrote today in the NY Times¹³²:

It's a stark juxtaposition: screening is good for women and bad for men. But just how different are these two cancer screening tests?

The answer is: not very. Neither is like the decision of whether or not to be treated for really high blood pressure. That's an easy one — do it. Instead, both breast and prostate cancer screening are really difficult calls, and the statistical differences between them are only of degrees. Reasonable individuals, in the same situation, could make different decisions based on their valuation of the benefits and harms of screening.

Personally, as a 56-year-old man, I choose not to be screened for prostate cancer (and, were I female, I believe I would choose not to be screened for breast cancer). Some of my patients have made the same choice, while others choose to be screened. That's O.K., because there is no single right answer.

Screening is like gambling: there are winners and there are losers.

However I have seen PCa patients with mets and DIC, which is not a pretty sight. The problem is that the question is NOT PSA. It is effective screening and what facilitates that. Somehow we have taken a crude but somewhat useful screening test and made it an all or nothing, a sine qua non. It is akin to throwing out the X ray because we really cannot use a plain film to diagnose a block or bleed well enough in a stroke. Then along came CAT and MRI scans. The issue must not be PSA, the issue must be proper screening. We are not there yet, and we are wasting too much time arguing over what we know to have problems.

The NY Times has noticed the following¹³³:

Healthy men should no longer receive a P.S.A. blood test to screen for prostate cancer because the test does not save lives and often leads to more tests and treatments that needlessly cause pain, impotence and incontinence in many, a key government health panel has decided.

The test measures a protein — prostate-specific antigen — that is released by prostate cells, and there is little doubt that it helps to identify the presence of cancerous cells in the prostate. But a vast majority of men with cancer of the prostate never suffer ill effects because the cancer is usually slow-growing. Even for men who do have fast-growing cancer, the P.S.A. test may not save them, since there is no proven benefit to earlier treatment of such invasive disease.

¹³² http://www.nytimes.com/2011/10/11/opinion/cancer-screenings-are-a-gamble.html?hp&_r=0

¹³³ <http://www.nytimes.com/2011/10/07/health/07prostate.html?hp>

We have shown the errors in many of the prior studies. But one should follow the logic as presented by the report. They state:

1. Healthy Men do not need the test.
2. PSA helps identifying cancer.
3. No benefit is provided by the PSA test to invasive cancer.

Now there is nothing wrong with any statement on its own. Taken together, however, it is total nonsense. Consider the following analysis of the statement:

1. How does one know that one is healthy? You have a test taken.
2. If PSA is effective in identifying PCa, albeit with some margin of error, then one has an indication, albeit one with some margin of error, but much less than total ignorance.
3. If one has PCa, then one is NOT healthy. If the test assist in ascertaining PCa, albeit in a somewhat faulty manner, then it assists in demonstrating the lack of health or the presence of disease.
4. Invasive or metastatic cancer is cancer which was not detected early has catastrophic results.
5. Thus, a man who did not have the test and was a priori considered healthy but was not is now subject to a devastating death!

Can one say that they still are following this nonsense? It would be doubtful. But why does one see this push now for eliminating the only test available at this time? Well it appears that it is the intent of the current Administration to apparently let the older and more costly be treated with less than appropriate care.

It is also interesting to see how the NY Times has edited their initial posting after perhaps much annoyance at their in my opinion illogical writing. The report to be presented by AHRQ, which according to HHS is:

The AHRQ Prevention and Care Management Portfolio fulfills AHRQ's Congressionally mandated role to support the U.S. Preventive Services Task Force (USPSTF). The USPSTF is an independent panel of non-Federal experts in prevention and evidence-based medicine and is composed of primary care providers (such as internists, pediatricians, family physicians, gynecologists/obstetricians, nurses, and health behavior specialists).

The USPSTF conducts scientific evidence reviews of a broad range of clinical preventive health care services (such as screening, counseling, and preventive medications) and develops recommendations for primary care clinicians and health systems. These recommendations are published in the form of "Recommendation Statements."

The AHRQ also drives Medicare allowances and thus it will most likely prohibit men from having this test. Thus, one would suspect like prohibition it will go underground. AHRQ has announced a study of comparative clinical effectiveness for treatment of prostate cancer. The study states:

Localized prostate cancer is a priority condition for comparative effectiveness research, and affects approximately 200,000 men in the United States each year. This study will examine the comparative effectiveness of management strategies for localized prostate cancer. Currently, few studies have directly compared the effectiveness of the different management modalities, including newer radiation and surgical techniques which have been widely adopted without proven benefit over older techniques.

The objectives are stated as:

- 1. To directly compare the **disease-free survival and treatment-related morbidity in men with localized prostate cancer treated by open radical prostatectomy, minimally-invasive (including laparoscopic and robotic) prostatectomy, 3D conformal radiation therapy, intensity-modulated radiation therapy, proton radiation therapy, and brachytherapy.***
- 2. To directly compare the prostate-cancer specific and global **quality of life (QOL) in men** with localized prostate cancer treated by the same treatment modalities.*

Part of the problem is defining localized cancer. It is not just the issue of invasiveness and tumor margins but it is more importantly the genetic makeup of the PCa cells. That is what should be addressed as well. Otherwise we are aggregating all cancers together. It is not as if we have no knowledge of what to look for, we do.

The major defect we argue is that the genomic profile will be more critical than say a Gleason score and if this becomes part of the HHS CCE protocol, they are doing a study with little if any merit. They are failing to consider the key profiling elements.

It is reasons like this that we are seriously concerned about the incompleteness and potentially false conclusions drawn for studies of this type.

The USPSTF has issued its dictum on PCa screening with PSA¹³⁴. It states:

The USPSTF recommends against PSA-based screening for prostate cancer (grade D recommendation).

This recommendation applies to men in the general U.S. population, regardless of age. This recommendation does not include the use of the PSA test for surveillance after diagnosis or

¹³⁴ <http://annals.org/article.aspx?articleid=1216568>

treatment of prostate cancer; the use of the PSA test for this indication is outside the scope of the USPSTF.

It continues:

Men with screen-detected cancer can potentially fall into 1 of 3 categories: those whose cancer will result in death despite early diagnosis and treatment, those who will have good outcomes in the absence of screening, and those for whom early diagnosis and treatment improves survival. Only randomized trials of screening allow an accurate estimate of the number of men who fall into the latter category. There is convincing evidence that the number of men who avoid dying of prostate cancer because of screening after 10 to 14 years is, at best, very small. Two major trials of PSA screening were considered by the USPSTF: the U.S. PLCO (Prostate, Lung, Colorectal, and Ovarian) Cancer Screening Trial and the ERSPC (European Randomized Study of Screening for Prostate Cancer).

The U.S. trial did not demonstrate any prostate cancer mortality reduction. The European trial found a reduction in prostate cancer deaths of approximately 1 death per 1000 men screened in a subgroup of men aged 55 to 69 years. This result was heavily influenced by the results of 2 countries; 5 of the 7 countries reporting results did not find a statistically significant reduction. All-cause mortality in the European trial was nearly identical in the screened and nonscreened groups.

The dissenting view stated¹³⁵:

Prostate cancer death was reduced by 21% in the screened compared with the control group and 29% after adjustment for noncompliance (5). The Task Force concluded that this decrease in prostate cancer–specific mortality amounted to few lives saved and did not outweigh ...

The recommendations of the USPSTF carry considerable weight with Medicare and other third-party insurers and could affect the health and lives of men at high risk for life-threatening disease. We believe that elimination of reimbursement for PSA testing would take us back to an era when prostate cancer was often discovered at advanced and incurable stages. At this point, we suggest that physicians review the evidence, follow the continuing dialogue closely, and individualize prostate cancer screening decisions on the basis of informed patient preferences.

Now for our comments:

1. We have discussed fatal flaws in our opinion in both studies relied upon. Simply they both used the old PSA threshold of 4 and did not include age dependency, percent free PSA and PSA velocity. In addition the European study had too great a time interval between tests.
2. No single PCa is alike. As we have been demonstrating for the past four years, the genetic makeup of PCa is complex and there are clearly certain specific markers for highly malignant PCa. By abandoning the test is throwing the baby out with the bathwater.

¹³⁵ <http://annals.org/article.aspx?articleid=1166178>

3. In my opinion this is a clearly age biased result, with the intent of removing care from the second highest cause of death amongst men.

4. Genetic makeup and family history are major drivers. PSA irregularities are one, along with PC3A testing, to ascertain PCa potential. Why eliminate it. The reason seems to be the cost of subsequent procedures, yet the Task Force argues it is the morbidity to the patient. Frankly morbidity in a competently performed procedure is less than a tooth extraction. Perhaps excess morbidity is more in the mind of the Task Force than reality.

What then is lost? We believe a great deal.

1. We are just beginning to understand the genetic makeup, just look at some of our recent postings, so that having the pool of data is indispensable. Having a genetic profile of multiple PCa would be the key to understanding the dynamics of PCa and its control.

2. What is the value of one life? If one has seen the agony of bone mets in a PCa patient, the results of DIC, and the loss of any dignity in the final days with catheter changes by a less than friendly "health care worker", the morbidity issue pales in comparison.

14.4 PSA EFFECTIVENESS AND SCREENING INTERVAL TIME

The recent NEJM article purporting to show that PSA screening saves lives has all sorts of issues, as do almost all of these¹³⁶. They state:

The principal screening test was measurement of the serum PSA level with the use of the Tandem-R/Tandem-E/Access assay (Hybritech). A positive test result, defined as a PSA value of 3.0 ng per milliliter or higher, was an indication for biopsy in most centers. Sextant prostatic biopsies were recommended for all men with positive test results; lateralized sextant biopsies⁴ were adopted in June 1996. Some exceptions to these procedures have been described previously.

That level of 3.0 is better than most, it is lower and has a higher false alarm rate and also higher detection probability on the ROC. They continue:

The median screening interval was 4.02 years. A total of 6963 prostate cancers were diagnosed in the screening group (cumulative incidence, 9.6%) and 5396 in the control group (cumulative incidence, 6.0%), with approximately 1000 additional cases of prostate cancer in each study group, as compared with our earlier analysis

Here is a problem if one reads this correctly, namely it was too long a screening period. **Again the question to be asked is what PSA level and what screening interval yields the best if any**

¹³⁶ http://www.nejm.org/doi/full/10.1056/NEJMoa1113135?query=featured_home#t=article see Schroder et al, Prostate-Cancer Mortality at 11 Years of Follow-up, NEJM, March 15, 2012.

survival. Then one can check the costs. The issue is really also one of looking at PSA as a progression over time including % Free as well.

We have argued herein based upon the well-known growth pattern of cancer cells that a one year maximum screening interval is necessary. Namely by having annual PSA tests we can see variations, velocities, and also have % Free PSA changes as well. The excessive 4.02 year interval is the limiting element of this study. Again here is another case of asking the wrong question but getting the right answer to the wrong question and extrapolating it improperly.

Now a Reuters report has some interesting comments¹³⁷:

Dr. Otis Brawley, chief medical officer of the American Cancer Society, said the European study is actually eight studies in eight countries, and only in Sweden and the Netherlands did PSA testing significantly reduce the risk of death from prostate cancer. "Screening saves lives if you live in the Netherlands and Sweden, but not the other six places," he told Reuters Health in a telephone interview.

One factor that may have skewed the Swedish data, he said, is that men who were screened were treated at an academic medical center, while men in the control group who developed cancer were treated elsewhere in the community. That alone might account for the lower mortality rate in the PSA population. In all, there were 299 prostate cancer deaths in the screening group compared to 462 in the control group that was not screened. Brawley said PSA testing is being widely promoted because "there's a huge profit in screening and treatment" for prostate cancer, even though most studies have failed to show that screening saves lives.

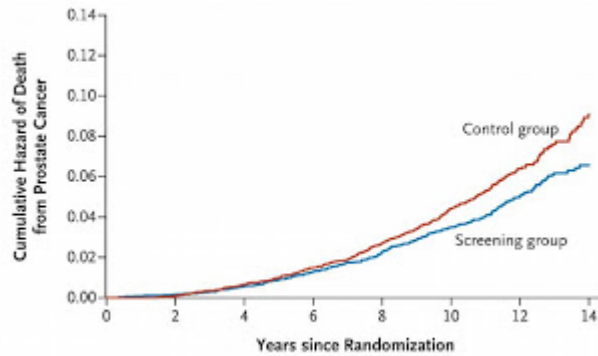
Strange, in my opinion, for ACS to advocate against screening as they seem to be saying above it is not what one would expect.

We all remember the discussion that the Government declared PSA tests as not effective. It was the same group which wanted to do away with mammograms. Most likely the same group which wants to do away with health care for anyone over 65, the current team in Washington that is.

Well NEJM just reported a different tale¹³⁸. Here it is:

¹³⁷ <http://www.reuters.com/article/2012/03/14/us-prostate-idUSBRE82D1DA20120314>

¹³⁸ http://www.nejm.org/doi/full/10.1056/NEJMoa1113135?query=featured_home#t=article see previous NEJM cite.



Namely it does save lives! Eureka, that is what we have been saying for the past four years, and now we have the data in spades.

Here is the test:

The principal screening test was measurement of the serum PSA level with the use of the Tandem-R/Tandem-E/Access assay (Hybritech). A positive test result defined as a PSA value of 3.0 ng per milliliter or higher, was an indication for biopsy in most centers. Sextant prostatic biopsies were recommended for all men with positive test results; lateralized sextant biopsies were adopted in June 1996. Some exceptions to these procedures have been described previously.

Yes a PSA cutoff of 3.0, not 4.0, and sextant biopsies. Today we would use 14 cores at a minimum, and saturation in many cases, say 24 cores, although it increases morbidity to a degree.

The end point was:

The primary end point of the trial was prostate-cancer mortality. We evaluated deaths among men in both the screening group and the control group in whom prostate cancer was diagnosed (including cases that were first diagnosed at autopsy), regardless of the official cause of death, as described previously. Data on overall mortality were collected by linkage to the national registries. Each trial center followed the common core protocol and provided key data to the joint independent data center every 6 months. The independent data monitoring committee received updates every 6 months according to a predefined monitoring and evaluation plan.

They conclude:

*The controversy regarding screening for prostate cancer has been renewed by the publication of the draft report of the U.S. Preventive Services Task Force, which after a literature-based analysis of benefits and harms recommended against the use of PSA testing in asymptomatic men. The report has been discussed in several Perspective articles in the Journal. **Clearly, the issue can be resolved only on the basis of evidence that considers both the advantages and disadvantages of screening, data that are not available at this time.** Our study **shows that the***

absolute effect of screening on the risk of death from prostate cancer increased in the intention-to-screen analysis from 0.71 to 1.07 deaths per 1000 men at a median of 11 years of follow-up, as compared with the initial results with a shorter follow-up period.

Clearly there is a benefit but clearly as we have previously stated they did not ask the correct question, which is:

"What should the PSA level be by age, race etc so as to have a decrease in survivability by some factor x?"

Notwithstanding, there is a clear benefit. The details of the benefit are yet to be determined but the conclusion is that the USPSTF conclusion is in error. The "Death Panel's conclusion is just that. And we have just begun!

Another example of how the Press gets things wrong. The JNCI paper we discussed before states¹³⁹:

Biopsying men with high PSA velocity but no other indication would lead to a large number of additional biopsies, with close to one in seven men being biopsied...We found no evidence to support the recommendation that men with high PSA velocity should be biopsied in the absence of other indications; this measure should not be included in practice guidelines.

Read this very carefully. Despite all the prior studies that are to the contrary, they state PSA velocity should not be the sole factor, specifically they state *high PSA velocity but no other indication*. Not that PSA velocity has any value at all. It is a conditional statement, so what are the conditions?

But the NY Times states¹⁴⁰:

The researchers, writing in the March 16 issue of The Journal of the National Cancer Institute, concluded that using P.S.A. velocity for prostate cancer detection is ineffective; that it leads to unnecessary biopsies and that references to it should be removed from professional guidelines and policy statements.

The Times continues:

Similarly, it is easy to demonstrate a statistical relationship between sharp rises in P.S.A. and cancer, but the correlation reveals no more information than is already available with a P.S.A. reading, a digital examination and a family history. It is irrelevant in deciding whether a biopsy is needed.

We had demonstrated on the basis of physiological, cellular and clinical data that one needs to

¹³⁹ <http://jnci.oxfordjournals.org/content/early/2011/02/24/jnci.djr028.abstract>

¹⁴⁰ <http://www.nytimes.com/2011/03/01/health/research/01prostate.html?hp>

track PSA over a long period. Looking at change is the concern. Change and rate of change are frankly the same. Yet the press all too often fails to grasp these less than subtle distinctions.

The final quote is:

But at this point, he is firmly against biopsies on the basis of velocity alone. “If your P.S.A. is in the normal range, you shouldn’t get a biopsy,” he said. “Changes or spikes in P.S.A. are not something to worry about if your P.S.A. is still normal.”

What if a patient has 2 first degree relatives each with an aggressive form of prostate cancer and who died in two years or less after diagnosis? Perhaps family history should play a part. Or is all of this a way to implement the comparative clinical effectiveness in the new health care law.

Will et al have published a paper in NEJM which concludes that radical prostatectomy in patients with prostate cancer does little to increase survivability. One could be concerned that this paper may be used beyond what in my opinion it should be. We shall describe the details and then present our opinions as to why there may be concern. Will et al conclude as follows¹⁴¹:

Patients had to be medically fit for radical prostatectomy and to have histologically confirmed, clinically localized prostate cancer (stage T1-T2NxM0 in the tumor–node–metastasis classification system according to the American Joint Committee on Cancer) of any grade diagnosed within the previous 12 months. Patients also had to have a PSA value of less than 50 ng per milliliter, an age of 75 years or less, negative results on a bone scan for metastatic disease, and a life expectancy of at least 10 years from the time of randomization. The study sites assessed eligibility on the basis of locally obtained PSA values and biopsy readings. After randomization, a central pathologist reviewed the biopsy and radical-prostatectomy specimens, and a central laboratory measured PSA...

Among men with localized prostate cancer detected during the early era of PSA testing, radical prostatectomy did not significantly reduce all-cause or prostate-cancer mortality, as compared with observation, through at least 12 years of follow-up. Absolute differences were less than 3 percentage points.

Let us first give some substance to the data and terms. This conclusion may have significant impact on many men who may very well be denied care under the ACA CCE rules if this paper stands and is interpreted without comment. Our objective is to analyze the paper to some extent but more importantly to raise an opinion which may re-interpret the results.

Let us first then define in some detail the AJCC terms¹⁴²:

- **T1:** tumor present, but not detectable clinically or with imaging

¹⁴¹ Will, T., et al, Radical Prostatectomy versus Observation for Localized Prostate Cancer, NEJM. July 19, 2012.

¹⁴² AJCC 6th Edition, 2002. Note D’Amico used the 5th Edition and thus we should be aware of a possible change. There was none.

- **T1a:** tumor was incidentally found in less than 5% of prostate tissue resected (for other reasons)
- **T1b:** tumor was incidentally found in greater than 5% of prostate tissue resected
- **T1c:** tumor was found in a needle biopsy performed due to an elevated serum PSA
- **T2:** the tumor can be felt (palpated) on examination, but has not spread outside the prostate
- **T2a:** the tumor is in half or less than half of one of the prostate gland's two lobes
- **T2b:** the tumor is in more than half of one lobe, but not both
- **T2c:** the tumor is in both lobes

Will et al go on to describe their patients as follows:

...13,022 men with prostate cancer,

5023 were eligible for enrollment. A total of

731 men (14.6%) agreed to participate and underwent randomization to

radical prostatectomy (364 men) or

observation (367).

The mean age was 67 years. Nearly one third of the patients were black; 85% reported full independence in activities of daily living.

The median PSA value was 7.8 ng per milliliter (mean, 10.1).

About 50% of the men had stage T1c disease (not palpable, detected by means of PSA testing), and about

25% had histologic scores of 7 or higher on the Gleason scale;

40% of the men had low-risk,

34% intermediate-risk, and

21% high-risk prostate cancer (about 5% had missing data).

On the basis of central pathological review, 48% of the patients had histologic scores of 7 or higher on the Gleason scale, and 66% had tumors in the intermediate-risk or high-risk categories.

D'Amico tumor risk score is used to differentiate in the above segmentation (low, intermediate, or high), which was based on tumor stage, the histologic score assigned by the local study site, and the PSA level. As D'Amico states¹⁴³:

In order to have the multivariable analysis results of the Cox proportional hazards regression model be applicable in the clinical setting for an individual patient, risk groups were defined. These risk groups were established from a review of the literature and were based on the known prognostic factors:

1. *PSA level,*
2. *biopsy Gleason score, and*
3. *1992 AJCC T stage.*

Patients with AJCC clinical T stage T1c, T2a and PSA level of 10 ng/mL or less and biopsy Gleason score of 6 or less have been identified to be at low risk (<25% at 5 years) for posttherapy PSA failure.

Conversely, patients with AJCC stage T2c disease or a PSA level of more than 20 ng/mL or a biopsy Gleason score of 8 or more have a risk higher than 50% at 5 years of posttherapy PSA failure.

The remaining patients with PSA levels higher than 10 and 20 ng/mL or lower, a biopsy Gleason score of 7, or AJCC clinical stage T2b have been found to have an intermediate risk (25%-50% at 5 years of posttherapy PSA failure).

Patients with AJCC clinical stage T1a, T1b were not managed using implant therapy because of the significant rate of urinary incontinence noted¹⁷ using this approach in patients with a history of a transurethral resection of the prostate. Therefore, patients with AJCC clinical stage T1a, T1b disease managed with RP or RT were excluded from the study to ensure statistically valid comparisons.

We summarize these categories below:

| Factor/Category | Stage | PSA | Gleason |
|------------------------|--------------|-------------------------------|----------------|
| Low | T1c or T2a | PSA Less Than 10 | 6 |
| Intermediate | T2b | 10-20<psa<20< p=""></psa<20<> | 7 |
| High | T2c | PSA More Than 20 | 8 or greater |

Furthermore from D'Amico et al we have the following:

¹⁴³ D'Amico et al, JAMA Network | JAMA: The Journal of the American Medical Association | Biochemical Outcome After Radical Prostatectomy, External Beam Radiation Therapy, or Interstitial Radiation Therapy for Clinically Localized Prostate Cancer, 1998.

Specifically, patients with biopsy Gleason score of 2 through 6 had no statistical difference in their estimates of PSA failure-free survival across all the treatment modalities evaluated in this study.

First, the comparison of PSA outcome for expectant management vs treatment is lacking. This comparison would be particularly relevant in the low-risk patients where 5-year PSA-progression rates numerically approximate the 10-year clinical-progression rates noted from expectant management series

Now returning to Till et al who concludes:

Among men with clinically localized prostate cancer that had been diagnosed after PSA testing came into practice, our study showed that radical prostatectomy did not reduce all-cause or prostate-cancer mortality, as compared with observation, through at least 12 years of follow-up.

The effect of radical prostatectomy on mortality did not vary according to age, race, self-reported performance status, or coexisting conditions, but our findings suggest that it may vary according to PSA value and possibly tumor risk.

Positive results were from multiple subgroup comparisons; the tests of interaction typically approached but did not reach significance and may therefore be due to chance.

Among men with PSA levels of 10 ng per milliliter or less, all-cause mortality was slightly lower at 12 years in the observation group than in the radical-prostatectomy group; prostate-cancer mortality in the observation group was 6%, with a nonsignificant absolute reduction of less than 1.0 percentage point in the radical-prostatectomy group.

Among men with low-risk disease, observation was associated with a nonsignificant reduction in all-cause and prostate cancer mortality, with no significant between-group difference in bone metastases.

*Among men with a PSA value that was greater than 10 ng per milliliter and possibly among those with intermediate-risk or high-risk prostate cancer (as determined according to the PSA value, local histologic findings, and stage), **absolute reductions in all-cause mortality with radical prostatectomy ranged from 6.7 to 13.2 percentage points.***

Thus there appears to be a reduction in survival. But what does that say? In high risk there very well may already be a metastasis, especially with such a high Gleason score.

Let us now make several observations. These are opinions which are subject to some further analysis but they in my opinion present several clear concerns and limitations.

1. No PSA velocity measurements are performed: Namely what if we used PSA velocity as a predictor, not just PSA. Gleason scores are ex post facto. Gleason of 8+ is a significant mortality

risk. Gleason of 6- is often rare. One does not record a Gleason 1 score for example and Gleason 3-4 is also infrequent.

2. No family histories were used: This is often the sine qua non determinant. If a 1st degree relative had an aggressive PCa then there is a high chance that the presenting patient will also have such. Also this test is free. Why it was not included is a concern.

3. No genetic analyses on tumors: The aggressiveness of the tumor is often demonstrated by the genes it expresses. Given the ease to do such tests and the limited numbers of patients it should have been incumbent on the study to have performed this analysis.

4. No attempt was made to ascertain that there existed a PCa stem cell. As with the genetic study not being done, there also was not attempt to ascertain any stem cell activity.

5. There is no attempt to define an aggressive form of PCa. One can admit the existence of indolent and aggressive. However, identifying what constitutes aggressive is questionable at this time. We have many genetic markers but there is not a bright line test. One can agree that a small percent are aggressive, and a large percent is indolent but again no test exists to determine this. Let us assume 5% are aggressive and 95% indolent. Further the 95% indolent will have no change in survival due to the PCa. However the 5% may very well have such a change. Furthermore if to get positive results from a prostatectomy with aggressive forms we must say perform it when the PSA velocity hits the 0.7 level, more than likely the patients coming to be seen are lost to the ravages of the disease, especially since they are performing tests on PSAs of 10. Thus the sample may be contaminated by results which fail to show any efficacy. That is 5% of all 3 groups will die and thus there will be de minimis efficacy. Just as we noted in the faulty prior studies, the wrong levels may very well have been chose, and thus the wrong question asked. The question should be; what PSA/PSA velocity tuples provide significant positive survival efficacy from prostatectomy.

6. What if one used PSA velocity and biopsied when it exceeded 0.7 per year. If that were the case then what percent would have an aggressive form of PCa?

Thus it is our belief that, although this paper does provide some valuable results, it fails in our opinion to understand and present many key factors essential for understanding and treating such a prevalent and deadly disease. Furthermore the alleged conclusions may actually create in my opinion a clear and present danger for those patients with family histories and genetically prone prostate cells. Namely under the new ACA regime, this may very well be used by the Government for refusal of service and result in substantial mortality and morbidity.

Now strangely NCI reports on FDA approval of a new test using percent free PSA but not PSA velocity. They state¹⁴⁴:

A PSA test score between 4 and 10 ng/mL often prompts physicians to recommend a prostate biopsy. Most biopsies from men with PSA scores in that range, however, reveal no cancer or

¹⁴⁴ <http://www.cancer.gov/ncicancerbulletin/071012/page8>

identify cancers that likely will never pose a health risk. And biopsies themselves have risks, including the risk of life-threatening infection.

The Access Hybritech p2PSA test measures a form of PSA called [-2] proPSA in the blood. Results from the test are combined with a PSA score and a measurement of free PSA to calculate the Prostate Health Index, or phi.

FDA approval was based on a clinical study of nearly 660 men, approximately half of whom had prostate cancer. In the study, the phi score was better able to distinguish between benign conditions and prostate cancer than the PSA score. The study also found that the probability of having prostate cancer detected following a biopsy rose as the phi score increased.

One does question the “life threatening” issues since in most cases of competent biopsies with proper preparation and execution the morbidity is low.

Ultimately, as with the other studies, perhaps the issue is the question which was asked. Perhaps the question should have been:

"What, if any, PSA measurement, Free PSA %, and PSA velocity, combined in some metric, will, with radical prostatectomy, increase survival?"

As with any research the key is always the question, not just the result. All too often failure to pose the proper question just reinforces poor judgment.

But in NEJM there is also an interesting and revealing editorial piece by Thompson and Tangren which states¹⁴⁵:

On the other hand, high-grade, aggressive prostate cancers usually have a lethal course if left untreated. Those of us who treat this disease are heartened to see men we treated years or decades ago for aggressive, high-grade cancer who remain cancer-free today. It is these men who are at greatest risk for death from cancer and who are most likely to benefit from therapy but whom we must treat effectively. Effective treatments often require multiple therapeutic approaches; for example, mortality is reduced among men with high-risk tumors in whom radiation therapy and surgery are augmented by androgen deprivation.

Prostate cancer is not a monolithic cancer but a spectrum of disease. The screening, detection, and treatment we provide must focus on cancers that matter, and future clinical trials must do so as well.

These authors indicate other issues with this study. We believe that valuable that this study may be there are many dimensions that need be addressed. Indeed as Thompson and Tangren state:

¹⁴⁵ <http://www.nejm.org/doi/full/10.1056/NEJMe1205012> see Thompson and Tangren, Prostate Cancer — Uncertainty and a Way Forward, NEJM, July 19, 2012.

Those of us who treat this disease are heartened to see men we treated years or decades ago for aggressive, high-grade cancer who remain cancer-free today.

Indeed, there are the many men with PSA of 10-15 with an indolent disease who will never die from the disease. There are also those men who one year have a PSA of 4 and then next 40, and are dead in three years. It would seem clear we are not dealing with the same disease and until we can determine via defects in pathways and the like what the difference is we are like creatures from Plato trying to identify the type on the basis of shadows on the walls of our caves.

In a recent piece by Dr. Messing of Rochester Medical Center, the article in Cell he states¹⁴⁶:

The objective of this study was to estimate the total number of patients who would be expected to present with metastatic (M1) prostate cancer (PC) in the modern US population in a given year if the age-specific and race-specific annual incidence rates of M1 PC were the same as the rates in the era before prostate-specific antigen (PSA) testing If the pre-PSA era rates were present in the modern US population, then the total number of men presenting with M1 PC would be approximately 3 times greater than the number actually observed.

Simply stated the evidence demonstrates that the use of PSA has reduced the number of M1 cases of PCa by a factor of 3. Messing as a clinician has obviously seen first-hand the results of not mitigating against this deadly disease. The bone mets, collapse of the spinal cord, the DIC results to name a few. Bone pain is excruciating. Thus anything that can be done to mitigate this is essential. On the NIH web site they have a compelling article describing the work¹⁴⁷.

The article states:

"PSA testing, for all its pluses and minuses and all that . . . permits you to catch the disease earlier," said lead researcher Dr. Edward Messing, chair of urology at the University of Rochester Medical Center in Rochester, N.Y. "These people are all going to die, they are going to die incredibly expensively and die miserably," he said, referring to the many men whose diagnoses would be delayed by not testing. "I don't know that all these people could be saved with PSA testing," but many could, he added. The report was published online July 30 in the journal Cancer.

Messing said the annual number of prostate cancer deaths dropped from about 42,000 in the 1990s to 28,000 now. "The only thing that can explain that is PSA early detection and treatment," he said.

Many cases of prostate cancer are not life-threatening, which is why testing is controversial. The U.S. Preventive Services Task Force (USPSTF) in May recommended against routine PSA

¹⁴⁶ <http://onlinelibrary.wiley.com/doi/10.1002/cnrc.27503/abstract> , see Scosyrev, Prostate-specific antigen screening for prostate cancer and the risk of overt metastatic disease at presentation, Cancer, July 30, 2012.

¹⁴⁷ http://www.nlm.nih.gov/medlineplus/news/fullstory_127726.html

screening, saying too many non-lethal cancers were being treated aggressively, exposing men who didn't need treatment to serious side effects such as impotence and urinary incontinence. But Messing disagreed with that advice. Condemning PSA testing "wasn't a brilliant conclusion," he said.

For the new study, Messing's team compared information from the U.S. Surveillance, Epidemiology, and End Results database for the years 1983 to 1985 -- immediately before widespread PSA testing started --- to data from 2006 through 2008. In the 2008 data, 8,000 cases of prostate cancer were diagnosed after the malignancy had spread to other parts of the body. Using these cases as a base, the researchers constructed a model that used data of advanced cancer diagnosed in the 1980s and predicted how many cases of advanced cancer would have been diagnosed in 2008 if PSA testing was not done. Their model showed instead of 8,000 actual cases in 2008, about 25,000 cases would have been diagnosed.

This is consistent with our arguments as well. This is telling especially today since HHS also announced all the "free" stuff for women while the USPSTF denies men equal protection. As we have said again and again, there will be some morbidly obese GS9 controlling the destiny and death of men.

14.5 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.

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 comment 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.

14.6 CANCER AND THE IMMUNE SYSTEM

There has been considerable work on melanoma and the immune system. We have somewhat neglected this effort here due mainly to a lack of understanding of how best to fit it into a system model, not because of any lack of importance.

As a recent work of Messina et al state:

Melanoma is notoriously insensitive to standard chemotherapy drugs that are widely used for other forms of cancer. However, melanoma is occasionally, and sometimes dramatically, responsive to immunotherapy. Immunotherapy is fast becoming an important part of the treatment armamentarium for advanced melanoma, but the degree of its clinical effectiveness varies among patients. In spite of the clinical success observed with antibodies against CTLA-4 and PD-1₄, cytokines (e.g., high-dose IL-2₄), as well as the adoptive transfer of tumor infiltrating lymphocytes_{4,5}, dramatic responses are observed in a minority of patients, while the majority of patients treated with those agents do not show significant clinical benefit. This current limitation underscores the need for the discovery of immune-related biomarkers and gene expression signatures that can identify/predict the likelihood of melanoma patients achieving a robust response of prolonged duration to immunotherapy.

They continue:

It is becoming widely accepted that immune cell infiltrates in human melanoma and other solid tumors have prognostic value^{6,7}. Many of these studies described the subset composition of the lymphocytic infiltrates that were either distributed diffusely within tumor parenchyma or localized diffusely to the peripheral rim of the mass and the tumor/stroma interface. In addition, recent attention has been focused on defining and potentially utilizing a so-called “immune score” based on the types and degrees of immune cell infiltrates for classification of human cancer.

Finally they conclude:

We have hypothesized that immune gene-related expression signatures can predict the presence of unique histological features of lymphoid cell infiltrates in solid tumor masses that correlate with better overall patient survival. In the current pilot study, we have shown that a 12-chemokine GES can accurately identify the presence of unique, TL-ELNs in metastatic melanoma, which also appear to be associated with better patient outcome.

Namely there are both immune system markers as well as targets for attack on melanoma. The early and seminal work of Rosenberg at NIH had approached melanoma and the immune system from an immune system direction, namely using the broad and general characteristics of the immune system to attack melanoma as foreign entities. The results have been mixed.

Perhaps improved results from the research will allow for a seamless integration of the pathway models developed herein and the operation of the immune system.

14.7 SUMMARY

As we had indicated at the beginning of this Chapter we are less focused upon summarizing the contents herein as we are in presenting a sense of the ongoing debate around PCa. On the one hand we have the powerful forces focused on PCa; with the assumption made by many that PCa is a single disease, while at the other extreme we have shown herein that PCa is a highly complex intracellular and intercellular disease which changes as it progresses through the body. The issues we face are:

1. What are the instigating factors in cell transformation? That is what causes the genes in the pathways to be modified. The specific instigating factors are still somewhat poorly understood.
2. How can we understand the initiation of malignant transformation at a distance by products sent out by the transformed cells?
3. What processes are involved with immune system responses to malignant progression? Is the immune system activated only after substantial malignancy has developed, or are their early stage markers one could look for.
4. What are early stage prognostic markers which could be used to ascertain indolent versus aggressive PCa using for example simple biopsy results or even less invasive properties.

5. Are there metabolic markers such as those used in PET scanning to assist in localizing early stage PCa?

The list of course can go on. But we hope that we have provided a view to PCa which can be used in a large scale and holistic manner.

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