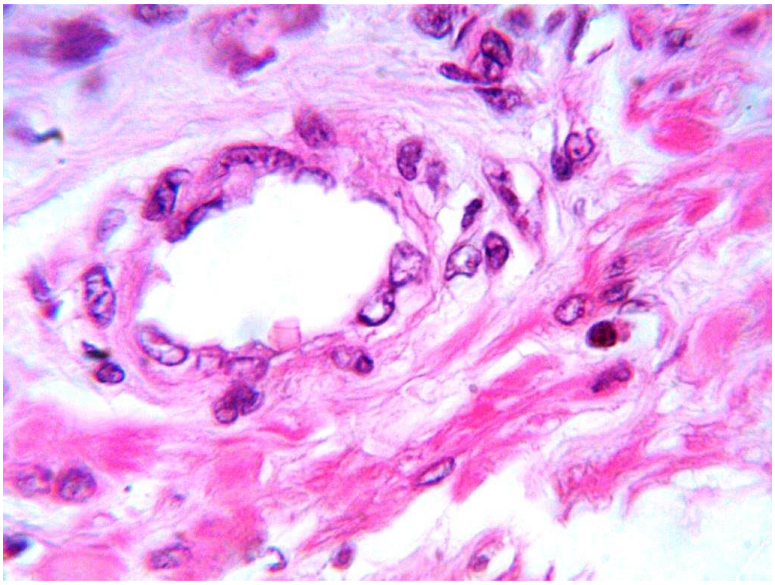

FIBROBLASTS AND CANCER: "THE WOUND THAT WOULD NOT HEAL"

ABSTRACT

Understanding cancers means not only understanding the cancer cells per se but also the tumor micro environment, the collection of other cells which support and protect the cancer cell mass. Attempts at blocking aberrant pathways or using immunotherapeutic methods have been seen to be blocked in a vast number of attempts. The blockage may very well be due to the TME as we discuss herein. Moreover, the fibroblast is an exceptionally interesting target for TME remediation and we examine that in detail.

TERRENCE MCGARTY

TGL 177, JUNE 2020

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Contents

1 INTRODUCTION

Cancer is much more complex than initially thought. Originally cancer was viewed in the context of the aberrant cell from a specific organ. Namely a cell from an organ had a genetic alteration and the result was aberrant proliferation and loss of function. Not only is a cell genetically changed to promote its aberrant growth but the cell apparently participates with its local environment to facilitate the process as well as developing a shield to prevent attack by the immune system or other entities. In a sense, the malignant cells manage to turn the very cells that protect the body, against the body they are to protect. Thus, the cancer is an amalgam of the aberrant cell and the collection of cells and entities about it that enable the malignant cells to not only survive but to become an entity unto itself. The implications are that any attempt to attack the cell alone may be thwarted by the other entities which are not only enablers but protectors.

In contrast, one can think of wound healing. In certain primitive animals we know that if you tale then, slice them mid body, head and tail, and then allow them to regrow, the net result is two identical regrown entities. There is no scar tissue, just a total regeneration of the original entity. As one goes up the chain in developed animals the ability to regenerate disappears and one is at best left with scar tissues. For the most part the scar tissue is developed by fibroblasts.

Thus, if one were to consider a set of malignant cells as an injury then the application of the fibroblasts and the formation of a putative scar may be a reasonable analogy. However, in this case the "scar" is a mutually self-sustaining entity which facilitates tumor growth and progression.

In this Note we examine some of the recent work regarding Fibroblasts and their association with malignancies. In a recent Consensus Statement, the authors have discussed the ability to gain an understanding of cancer associated fibroblasts. As Sahai et al note:

Cancer-associated fibroblasts (CAFs) are a key component of the tumour microenvironment with diverse functions, including matrix deposition and remodelling, extensive reciprocal signalling interactions with cancer cells and crosstalk with infiltrating leukocytes. As such, they are a potential target for optimizing therapeutic strategies against cancer. However, many challenges are present in ongoing attempts to modulate CAFs for therapeutic benefit. These include limitations in our understanding of the origin of CAFs and heterogeneity in CAF function, with it being desirable to retain some antitumorigenic functions.

This note is a focus on CAFs as well as other elements of the tumor micro environment. The above remarks from the recent Consensus Statement highlights another set of targets for the treatment of cancers. We have experienced an explosion of ways to target the cancer cell itself. However we know time and again that for a majority of the patients the response is lacking. We address that issue herein where we argue that the TME and its associated cells are the next barrier to breach.

1.1 FUNDAMENTAL ISSUES

Let us begin with the overall stroma, or in Greek, στρομα (packing bed, sack, bedding). The stroma is the collection of elements that surround and support a cell. In cancer cells the stroma can in a sense be identified as the extracellular matrix, plus the cells supporting this such as fibroblasts.

As Maddaluno et al note in their review of fibroblast growth factors, "FGF", they note:

Tissue injury initiates a complex repair process, which in some organisms can lead to the complete regeneration of a tissue. In mammals, However, the repair of most organs is imperfect and results in scar formation. Both regeneration and repair are orchestrated by a highly coordinated interplay of different growth factors and cytokines. Among the key players are the fibroblast growth factors (FGFs), which control the migration, proliferation, differentiation and survival of different cell types. In addition, FGFs influence the expression of other factors involved in the regenerative response. Here, we summarize current knowledge on the roles of endogenous FGFs in regeneration and repair in different organisms and in different tissues and organs. Gaining a better understanding of these FGF activities is important for appropriate modulation of FGF signaling after injury to prevent impaired healing and to promote organ regeneration in humans.

Thus, FGF in one sense are important in understanding the regrowth of cells injured and also in turn their use in the explosive growth of tumor cells. Cancer cells have a somewhat unique capability in engaging the resources of a multiplicity of other cells, often one which would normally be protective against invaders, and using these cells to assist in its own proliferation. The amalgam of these cells is called the tumor micro environment, TME.

The TME is often overlooked in histological studies where the focus is on the morphology of the aberrant source cells. For example, in examining a thyroid malignancy, one all too often examines the nucleus and nucleolus of the originating thyroid cells. and is the growth of macrophages or fibroblasts which have become an integral part of the malignancy. The same is somewhat true of melanomas and prostate cancers and a wealth of other solid malignancies.

As Biffi and Tuveson have noted:

Stromal cells constitute the tumor microenvironment (TME), a niche where neoplastic cells reside and progress. While the genetic and epigenetic drivers of cancer cells have been extensively investigated, the mechanisms governing the recruitment and activation of a major stromal cell type, cancer-associated fibroblasts (CAFs), are largely unknown.

Investigating the origin and developmental lineage of CAFs is essential for determining their functions and designing means to impede their tumor-supportive roles.

CAFs may be globally viewed as the chief architects of the TME due to their multiple functions. Indeed, they are considered the major source of extracellular matrix components that alter

physical-chemical properties, concomitantly impairing vascular function and, therefore, drug delivery.

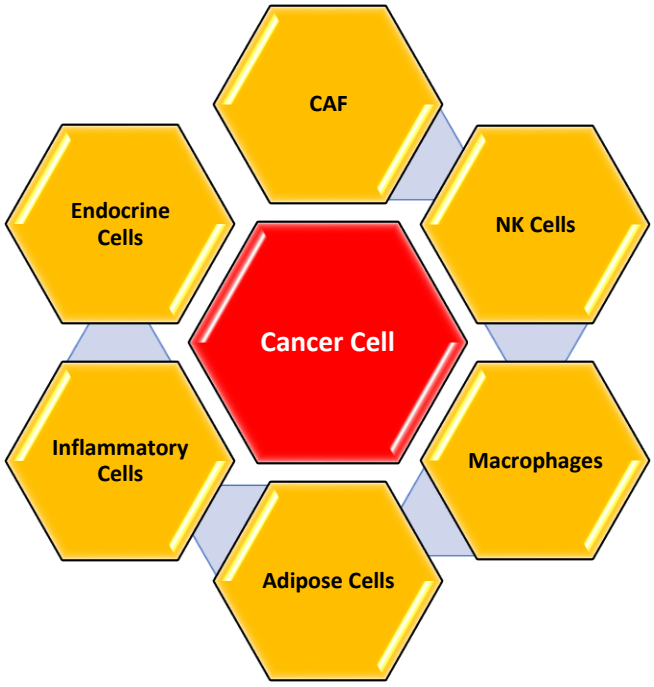
Furthermore, CAFs secrete paracrine ligands that promote tumor growth, angiogenesis and drug resistance, and directly blunt T cell cytotoxicity while recruiting immunosuppressive populations. Therefore, a multitude of preclinical and clinical studies have attempted to antagonize CAFs as a treatment modality for cancer.

However, the classical view of uniformly pro-tumorigenic CAFs has been modified by the recent identification of subsets with tumor-suppressive properties. This new appreciation that CAFs are a heterogenous population in the TME prompts a reevaluation of CAF identities and functions in efforts to develop more effective therapies.

Changes can be made to cancer cells such as the epithelial to mesenchymal transition process[1].

1.2 Basic Observations

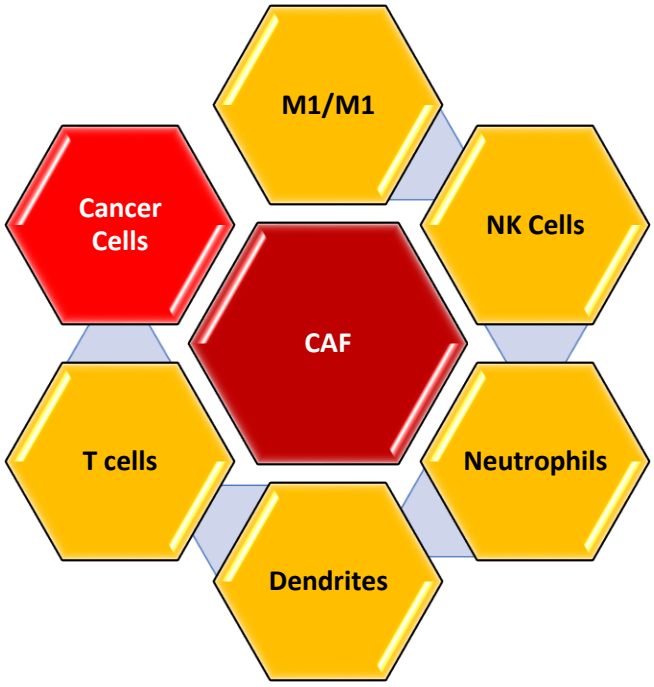
We can make two basic observations. The first will be the interactions with the cancer cell and the other elements of the tumor micro environment, TME. We show this below:



The above is a simple demonstrative that shows that the cancer cell can control and be controlled by a significant selection of the TME.

In a similar fashion we can look at the CAF and see how it influences the immune environment. This is a critical observation. We show this below:

[1] https://www.researchgate.net/publication/330222973_EMT_and_Cancers



What is critical to note is that the CAF can control a large set of the immune system. This in effect tends then to block any immunotherapeutic approach to mitigating the cancer cells.

1.3 Overview

The previous section details some of the key points we will discuss. The driver herein is the Consensus Statement recently issued and referred to herein regarding CAFs. We will present the following:

1. Fibroblasts and FGF. These are the current cells and the growth factors associated somewhat with them. FGF are a large group and a few are fibroblast related whereas the name encompasses a larger set of cells.

2. The ECM and TME: The ECM is the mass of extracellular proteins and the tumor micro environment is the collection of other cells which make up the total tumor mass.

3. Adipocytes: This will be a discussion on another set of cells which are frequently forgotten, The often play a key role in the malignant process.

4. ECM signalling discusses how the TME uses the ECM for intercellular signalling of a holistically defined tumor mass.

5. There is a detailed discussion of the current understanding of the CAF

6. The immune elements are discussed and a specific focus on immunotherapeutic issues.

7. We then consider a specific malignancy, prostate cancer, and the issues of CAF.

Overall, understanding the TME and the elements that make it up is critical to understand the "system" that functions in many cancers. Hematopoietic cancers may be different in that they present themselves bare of any significant TME. On the otherhand somatic cancers often have developed a significant protective environment to sustain themselves.

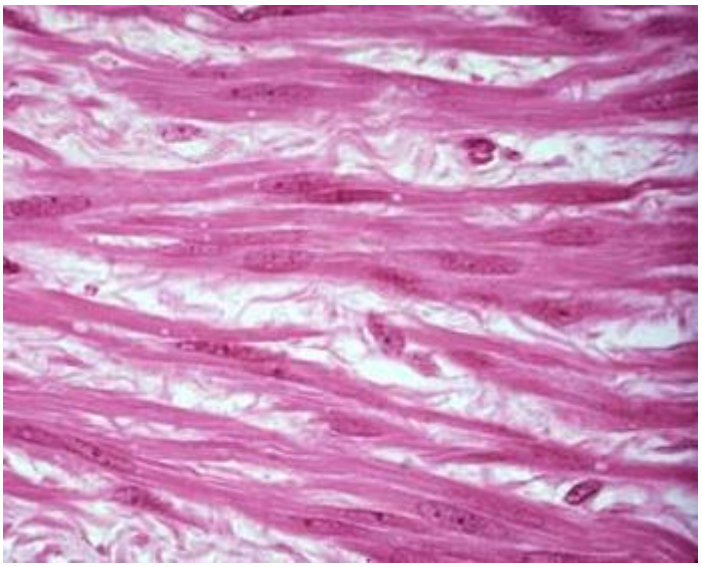
Fundamentally, we must understand cancers as a set of complex interacting systems. Instead of just targeting one aberrant element of another we must target the system as a whole. Yet to do that we must first truly understand the system. Our goal in the Note is to start with a step in that process.

2 FIBROBLASTS

Fibroblasts are common cells that generally do not form any specific functioning collection of cells. The fibroblast is resident in the stroma of most organs.

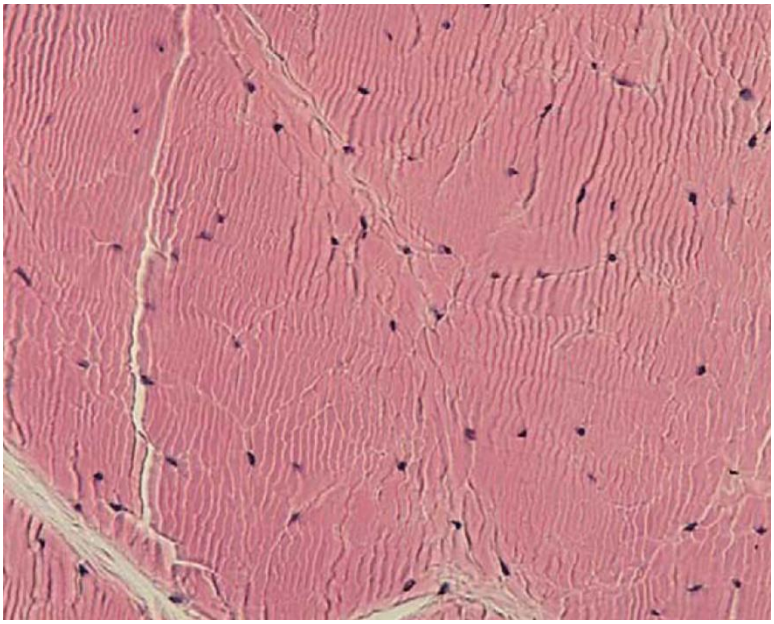
2.1 HISTOLOGY

We start by examining the fibroblasts histologically. Fibroblasts seem to be almost universal and part of the vast connective matrix. An example is shown below[2]:

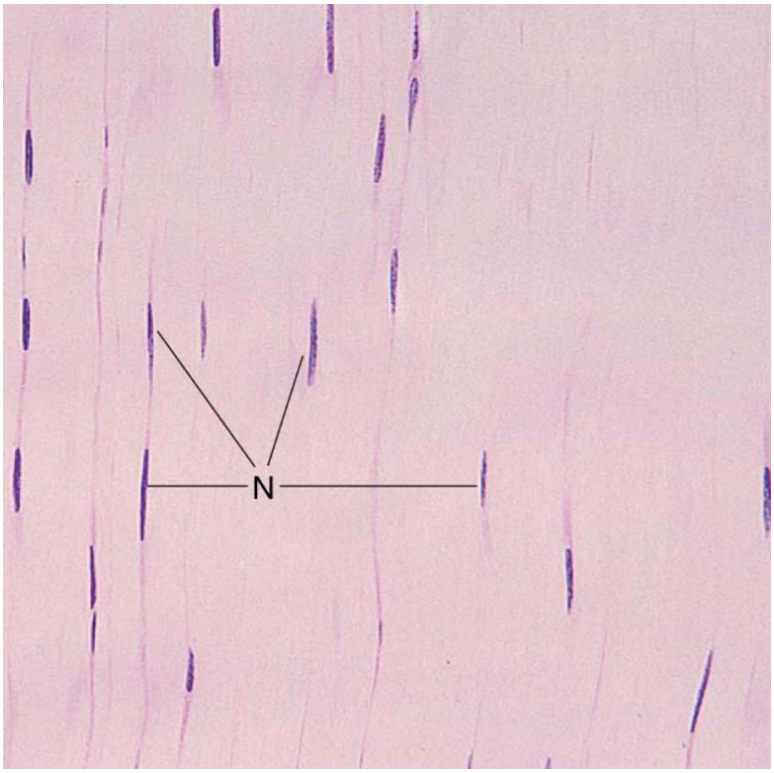


The following are from Gartner and Hiatt:

[2] http://www.meddean.luc.edu/lumen/MedEd/Histo/frames/h_frame7.html



and the following is another example from the same source.



In both of the above cases the fibroblasts are elongated, prominent nuclei and somewhat clear protoplasm. All of the above fibroblasts and long tear shaped cells with prominent nuclei. They generally are unorganized and have a clear cytoplasm.

2.2 FIBROBLAST FUNCTIONS

From NCBI we have[3]:

The fibroblast is one of the most abundant cell types present in the stroma. It has a variety of functions and composes the basic framework for tissues and organs. Under homeostasis, this cell is responsible for maintaining the extracellular matrix (ECM). During stress, fibroblasts adapt to their environment and have the ability to respond and send local signals. In times of injury, the fibroblast can transform phenotypes and synthesize the building blocks necessary to replace wounded tissue. During pathologic states, the extracellular matrix gets generated in excessive quantities, and collagen is deposited in a dysregulated manner often causing irreversible organ dysfunction or disfiguring appearance….

Fibroblasts are the most common cell type represented in connective tissue. These cells produce a diverse group of products including collagen type I, III, and IV, proteoglycans, fibronectin, laminins, glycosaminoglycans, metalloproteinases, and even prostaglandins. In the adult body, fibroblasts remain in a quiescent form until stimuli activate protein synthesis and contractile mechanisms.

These cells synthesize reorganize the ECM found in the skin, lung, heart, kidney, liver, eye, and other organs. The ECM is in constant communication with the surrounding cells as fibroblasts can secrete and respond to both autocrine and paracrine signals. Matrix reorganization occurs through a process of degradation and crosslinking enzymes, produced by fibroblasts, that are activated and regulated by pro-inflammatory cytokines and growth factors. Transcription growth factor-alpha and beta (TGF-A and TGF-B), platelet-derived growth factor (PDGF), granulocyte-macrophage colony-stimulating factor (GM-CSF), epidermal growth factor (EGF), and tumor necrosis factor (TNF) all have implications in fibroblast regulation.

The relationship with the ECM is an important factor especially when we examine its role in various cancers. They continue:

Fibroblasts are a diverse group of cells. Within one organ system, there can be a great variety of functions. Within the integument, dermal fibroblasts in different locations have separate roles. The superficially located lineage involves the formation of the hair follicle and is responsible for reepithelization during wound healing; the deeper lineage is responsible for ECM generation.

Fibroblasts are known for their plasticity; adipocytes, pericytes, endothelial and epithelial cells, otherwise known as terminally differentiated cells, can de-differentiate into fibroblasts.

Stimulation of fibroblasts further increases susceptibility to epigenetic modifications. The ability of fibroblasts to transform is partly due to the variety of cell-surface adhesion receptors (integrins, syndecans, cadherins) that facilitate the communication of fibroblasts with their surroundings. One of these well-described fibroblast transformations is the transformation of fibroblast into the myofibroblast.

[3] https://www.ncbi.nlm.nih.gov/books/NBK541065/

Myofibroblasts are present in both healthy and pathologic tissues and contain features of fibroblasts and smooth muscle cells. These cells work in conjunction with vascular endothelial cells to form granulation tissue during times of wound healing.

In the following, many of the cancer related involvements of fibroblasts will focus on the transitioned myofibroblast.

2.3 SCARS AND MARKERS

Identifying fibroblasts are generally done histologically by visible inspection but they also can be further classified by surface markers. Now this is discussed in Ziani et al who note:

Fibroblasts are spindle-shaped, non-epithelial (cytokeratin negative, E-cadherin negative), non-endothelial (CD31 negative) and non-immune (CD45 negative) cells of a mesenchymal lineage origin (vimentin+). In normal tissue, fibroblasts are usually considered as resting/ quiescent cells with negligible metabolic and transcriptional activities, but with the ability to respond to growth factors to become activated.

This is an exceptionally short and clear description. The fibroblasts are cells somewhat on their own and are interstitial to organ focused cells. The lack of E cadherin allows them to have substantial mobility.

During this activation process, fibroblasts exhibit contractile activity, exert physical forces to modify tissue architecture, acquire proliferation and migration properties and become transcriptionally active leading to the secretion of several factors (cytokines, chemokines, etc.) and ECM components.

The ability of resting fibroblasts to become activated was first observed in the context of wound healing and subsequently in pathologic conditions such as acute or chronic inflammation or tissue fibrosis (a chronic wound healing response).

This chronic tissue repair response also occurs in the context of cancer, considered as a "wound that never heals".

This concept of wound healing is a significant driver of understanding how fibroblasts play such a role in cancers. Wound healing is the process in humans of repairing damaged organs and in turn cells. It is a tissue repair attempt, albeit one poorly accomplished, yet its ultimate protective result allows and facilitates a malignant growth.

Indeed, emergence and/or accumulation of cancer cells in a given tissue represent a tissue injury, imitating a chronic wound healing response toward the tumor cells, also known as tumor fibrosis or desmoplastic reaction.

Consequently, major players in tumor fibrotic microenvironment include activated fibroblasts, termed cancer-associated fibroblasts (CAFs), which represent one of the most abundant

stromal cell types of several carcinomas including breast, prostate, pancreatic, esophageal, and colon cancers while CAFs are less abundant, but still present, in other neoplasias including ovarian, melanoma, or renal tumors. For example, in pancreatic cancer, 60–70% of the tumor tissue is composed of a desmoplastic stroma characterized by extensive collagen deposition and activated CAFs.

Now it is the CAF that we will focus upon. However, the key issue to note is that the fibroblasts play a significant role in wound repair. As Kumar et al note:

Several cell types proliferate during tissue repair. These include the remnants of the injured tissue (which attempt to restore normal structure), vascular endothelial cells (to create new vessels that provide the nutrients needed for the repair process), and ***fibroblasts (the source of the fibrous tissue that forms the scar to fill defects that cannot be corrected by regeneration).***

The ability of tissues to repair themselves is determined, in part, by their intrinsic proliferative capacity. In some tissues (sometimes called labile tissues), cells are constantly being lost and must be continually replaced by new cells that are derived from tissue stem cells and rapidly proliferating immature progenitors. These types of tissues include hematopoietic cells in the bone marrow and many surface epithelia, such as the basal layers of the squamous epithelia of the skin, oral cavity, vagina, and cervix; the cuboidal epithelia of the ducts draining exocrine organs (e.g., salivary glands, pancreas, biliary tract); the columnar epithelium of the gastrointestinal tract, uterus, and fallopian tubes; and the transitional epithelium of the urinary tract. These tissues can readily regenerate after injury as long as the pool of stem cells is preserved.

Other tissues (called stable tissues) are made up of cells that are normally in the G0 stage of the cell cycle and hence not proliferating, but they are capable of dividing in response to injury or loss of tissue mass. These tissues include the parenchyma of most solid organs, such as liver, kidney, and pancreas. ***Endothelial cells, fibroblasts, and smooth muscle cells are also normally quiescent but can proliferate in response to growth factors, a reaction that is particularly important in wound healing.***

Now they continue on the process of developing a scar, or scar tissue as follows:

1. Within minutes after injury, a hemostatic plug comprised of platelets is formed, which stops bleeding and provides a scaffold for infiltrating inflammatory cells.

2. Inflammation. This step is comprised of the typical acute and chronic inflammatory responses. Breakdown products of complement activation, chemokines released from activated platelets, and other mediators produced at the site of injury function as chemotactic agents to recruit neutrophils and then monocytes during the next 6 to 48 hours. As described earlier, these inflammatory cells eliminate the offending agents, such as microbes that may have entered through the wound, and clear the debris. Macrophages are the central cellular players in the repair process—M1 macrophages clear microbes and necrotic tissue and promote inflammation in a positive feedback loop, and M2 macrophages produce growth factors that stimulate the proliferation of many cell types in the next stage of repair. As the injurious agents and necrotic

cells are cleared, the inflammation resolves; how this inflammatory flame is extinguished in most situations of injury is still not well defined.

3. Cell proliferation. In the next stage, which takes up to 10 days, several cell types, including epithelial cells, endothelial and other vascular cells, and fibroblasts, proliferate and migrate to close the now-clean wound. Each cell type serves unique functions.

a. *Epithelial cells respond to locally produced growth factors and migrate over the wound to cover it.*

b. *Endothelial and other vascular cells proliferate to form new blood vessels, a process known as angiogenesis. Because of the importance of this process in physiologic host responses and in many pathologic conditions, it is described in more detail later.*

c. ***Fibroblasts proliferate and migrate into the site of injury and lay down collagen fibers that form the scar.***

d. ***The combination of proliferating fibroblasts, loose connective tissue, new blood vessels and scattered chronic inflammatory cells, forms a type of tissue that is unique to healing wounds and is called granulation tissue. This term derives from its pink, soft, granular gross appearance, such as that seen beneath the scab of a skin wound.***

4. Remodeling. The connective tissue that has been deposited by fibroblasts is reorganized to produce the stable fibrous scar. This process begins 2 to 3 weeks after injury and may continue for months or years.

Now as we noted earlier, this process seems to occur with the introduction of malignant cells as well. Unlike a normal benign scar, However, a malignant scar or tumor, uses the same elements but it does so in a manner to protect itself. It uses the fibroblasts as a tool for protection.

3 FGF

Growth factors are many in number and are often key players in the proliferation of cancers. Fibroblast growth factors, FGF, are broadly functioning growth factors. They obtained their names from their initial discovery on fibroblasts but they are more common than just that. They often activate a variety of kinase pathways in cells and play a significant role in multiple malignancies.

As Yun et al have noted regarding the historical linkage to fibroblasts as follows:

Fibroblast growth factor (FGF) is a representative growth factor which has shown the potential effects on the repair and regeneration of tissues.

It was originally identified as a protein capable of promoting fibroblast proliferation and is now known to comprise 22 members.

FGFs exert multiple functions through the binding into and activation of fibroblast growth factor receptors (FGFRs), and the main signaling through the stimulation of FGFRs is the RAS/MAP kinase pathway. With their potential biological functions, FGFs have been utilized for the regeneration of damaged tissues, including skin, blood vessel, muscle, adipose, tendon/ligament, cartilage, bone, tooth, and nerve.

Then, the prospective source of FGF for the tissue regeneration is used with recombinant human FGF family. In fact, many previous studies administered the FGFs directly to the wound sites, like other growth factors. However, free-FGFs are readily degradable in vivo, leading to loss of biological activity and functions. To gain satisfactory performance, FGFs are adsorbed onto or encapsulated within materials to secure biological activity in a sustained and controllable manner. Although many types of materials have been developed to carry FGFs and elicit their therapeutic efficacy in vitro and in vivo, more sustained, controlled, and targeted delivering system still remain a challenge

Thus, FGFR obtain their name in an historical manner based upon the vehicle in which they were first identified yet have a wide range of functionality.

3.1 FGF FUNCTIONS

We now want to examine some of the functionality of the FGF.As Teishima et al have recently noticed in a discussion on prostate cancer:

Fibroblast growth factors (FGFs) and FGF receptors (FGFRs) play an important role in the maintenance of tissue homeostasis and the development and differentiation of prostate tissue through epithelial-stromal interactions. Aberrations of this signaling are linked to the development and progression of prostate cancer (PCa). The FGF family includes two subfamilies, paracrine FGFs and endocrine FGFs.

Paracrine FGFs directly bind the extracellular domain of FGFRs and act as a growth factor through the activation of tyrosine kinase signaling.

Endocrine FGFs have a low affinity of heparin/heparan sulfate and are easy to circulate in serum. Their biological function is exerted as both a growth factor binding FGFRs with co-receptors and as an endocrine molecule.

Many studies have demonstrated the significance of these FGFs and FGFRs in the development and progression of PCa. Herein, we discuss the current knowledge regarding the role of FGFs and FGFRs—including paracrine FGFs, endocrine FGFs, and FGFRs—in the development and progression of PCa, focusing on the representative molecules in each subfamily.

Thus, the FGF can significantly influence other cells and this is especially the case in cancer cells. FGF are but one class of growth factors[4]. Importantly the FGF act as both paracrine and endocrine. They can act closely and also at a distance. As we shall also note, this is the case for a variety of the cells in the EMT.

As will be noted, there are 18 such growth factors all possessing the ability to activate cells in a variety of ways; paracrine and endocrine. Now as Wesche et al have noted, the structure and complexity of the FGF family is also significant:

The FGF family consists of 18 ligands that bind to four homologous high-affinity FGFRs (FGFR1–FGFR4). The FGFs are secreted polypeptidic growth factors that bind to receptors expressed at the cell surface of target cells.

Most FGFs have signal sequences for secretion, except FGF1 and FGF2 that utilize a non-classical secretion pathway circumventing the ER (endoplasmic reticulum). In addition to the 18 secreted ligands that bind to cell-surface receptors, four members of the FGF family, the FHFs (FGF homologous factors), are not secreted and act intracellularly. The FGFRs have an overall structure similar to most RTKs. They are single-pass transmembrane proteins that consist of an extracellular part that binds FGF ligands, a transmembrane domain and an intracellular tyrosine kinase domain that transmits the signal to the interior of the cell.

The intracellular kinase domain is similar to the VEGFR and PDGFR kinases in that it has an insert, resulting in a split kinase domain. The extracellular part is composed of three Ig-like domains (I–III) with an acidic, serine-rich region between domains I and II (termed the acid box). The first Ig-like domain is, together with the acid box, thought to play a role in receptor autoinhibition.

Domains II and III constitute the FGF ligand-binding site. In FGFR1–3, alternative splicing in Ig-like domain III creates isoforms with different ligand-binding specificities (FGFR1 IIIb–FGFR3 IIIb and FGFR1 IIIc–FGFR3 IIIc) [12,23]. The FGFR IIIb isoforms are predominantly epithelial and the IIIc isoforms are predominantly mesenchymal, with their corresponding ligands only activating either the epithelial or mesenchymal isoforms, except FGF1 which binds

[4] https://www.researchgate.net/publication/329702571_Growth_Factors_Pathways_and_Cancers

all receptor isoforms. Thus, paracrine signalling is achieved by, for instance, epithelial cells producing ligands that only activate the corresponding mesenchymal FGFR IIIc isoforms, and vice versa.

FGFs also bind to low-affinity receptors present on most cells, the HSPGs (heparan sulfate proteoglycans). HSPGs consist of a proteoglycan core that binds two or three linear polysaccharides (heparan sulfate chains). The FGFs bind to the negatively charged polysaccharides through electrostatic interactions. HSPGs both protect the ligands from degradation and are also involved in the complex formation between the FGFs and the FGFRs. Binding of FGFs to the receptors forces the dimerization of a ternary complex consisting of FGF, FGFR and heparan sulfate

From Yun et al we have the following summary Table as modified:

Function	***Subfamily related to the function***	***Target cell***
Cell Proliferation	FGF1, FGF2 FGF4	Preadipocyte Endothelial cell, epithelial cell, fibroblast cell, neural stem cell Trophoblast stem cell
Cell Proliferation	FGF7, FGF10	Epithelial cell
Cell Proliferation	FGF18	Osteoblast, chondrocytes, osteoclast
Cell Migration	FGF2	Astrocyte, myogenic cell
Cell Migration	FGF4	Myogenic cell
Cell Migration	FGF7	Epithelial cell, keratinocyte
Cell Migration	FGF8	Neural crest cell
Cell Differentiation	FGF1, FGF2	Neuroepithelial
Cell Differentiation	FGF7	Keratinocyte
Cell Differentiation	FGF20	Monkey stem cell
Angiogenesis	FGF1, FGF2	Endothelial cell

These are a brief summary of the FGR functions and targets. We shall examine these in the context of the fibroblast as well as the TME in toto.

3.2 FGFR

The FGF receptor, FGR plays a significant role in tumor development. The receptors are what takes the growth factor and then allows it to make the cell perform in a specific manner. As Acevedo et al have noted:

Fibroblast growth factor receptors (FGFRs) comprise a subfamily of receptor tyrosine kinases (RTKs) that are master regulators of a broad spectrum of cellular and developmental processes, including apoptosis, proliferation, migration and angiogenesis. Due to their broad impact,

FGFRs and other RTKs are highly regulated and normally only basally active. Deregulation of FGFR signaling by activating mutations or ligand/receptor overexpression could allow these receptors to become constitutively active, leading to cancer development, including both hematopoietic and solid tumors, such as breast, bladder and prostate carcinomas.

Aberrant expression of multiple FGF family members and their cognate receptors are found in multiple cancers, including PCa, providing a strong indication of their role in neoplastic progression. While FGFR2 signaling is key in regulating both prostate morphogenesis and homeostasis, FGFR1 signaling has been more tightly correlated with PCa progression, evidenced by elevated expression of FGFR1 and some of its ligands in both human PCa and mouse prostate tumor models, such as SV40 T/t antigen (T/tag)- based TRAMP.

There have been a number of studies to date of the expression of FGFR1 in human PCa.12,16-19 Both our studies12 and those of Hamaguchi et al.20 demonstrate that in benign prostate glands, staining is seen primarily in cells in a basal location within the gland (encompassing basal cells, transit amplifying cells and prostatic progenitor cells) although the exact cell type expressing FGFR1 is unclear. All studies to date using immunohistochemistry (IHC) have shown increased expression of FGFR1 in PCa. …Thus, it is unequivocal that FGFR1 is increased in PCa. The linkage of FGFR1 to outcome is less clear.

In the case of melanomas, for example, Metzner et al have noted:

Cutaneous melanoma is a tumor with rising incidence and a very poor prognosis at the disseminated stage. Melanomas are characterized by frequent mutations in BRAF and also by overexpression of fibroblast growth factor 2 (FGF2), offering opportunities for therapeutic intervention. We investigated inhibition of FGF signaling and its combination with dacarbazine or BRAF inhibitors as an antitumor strategy in melanoma.

The majority of melanoma cell lines displayed overexpression of FGF2 but also FGF5 and FGF18 together with different isoforms of FGF receptors (FGFRs) Blockade of FGF signals with dominant-negative receptor constructs (dnFGFR1, 3, or 4) or small-molecule inhibitors (SU5402 and PD166866) reduced melanoma cell proliferation, colony formation, as well as anchorage-independent growth, and increased apoptosis.

DnFGFR constructs also significantly inhibited tumor growth in vivo. ***Combination of FGF inhibitors with dacarbazine showed additive or antagonistic effects, whereas synergistic drug interaction was observed when combining FGFR inhibition with the multikinase/BRAF inhibitor sorafenib or the V600E mutant-specific BRAF inhibitor RG7204.*** *In conclusion, FGFR inhibition has antitumor effects against melanoma cells in vitro and in vivo. Combination with BRAF inhibition offers a potential for synergistic antimelanoma effects and represents a promising therapeutic strategy against advanced melanoma. ….*

The use of BRAF inhibitors has been shown to have significant impact on melanomas, but not universally. This discussion points again towards the influence of the TME and particularly the FGF. They continue:

Overexpression of growth and survival-promoting factors is an important hallmark of neoplastic cells and a major driving force for tumor progression and dissemination. Expression of fibroblast growth factor 2 (FGF2) has been identified as an important characteristic of melanoma cells in contrast to normal melanocytes and has been linked to tumor progression in melanoma and multiple other malignancies .

The role of other FGFs is widely unexplored in melanoma so far. FGFs constitute a structurally conserved family of polypeptide growth factors, with 22 members in humans. FGFs transduce signals through binding to transmembrane receptor tyrosine kinases, named FGF receptors (FGFR1–4), and also bind with lower affinity to heparin-like glycosaminoglycans of the extracellular matrix. After ligand binding, FGFRs activate major cellular growth and survival pathways including, for example, mitogen-activated protein kinase and phosphoinositide 3-kinase signal cascades.

In addition, in a review paper by Wesche et al the authors summarize a multiplicity of cancers related to the FGFR. Specifically:

i. Breast
ii. Bladder
iii. Prostate
iv. Endometrial
v. Lung
vi. Myeloma
vii. Rhabdomyosarcoma
viii. EMS/SCLL (Leukemia)

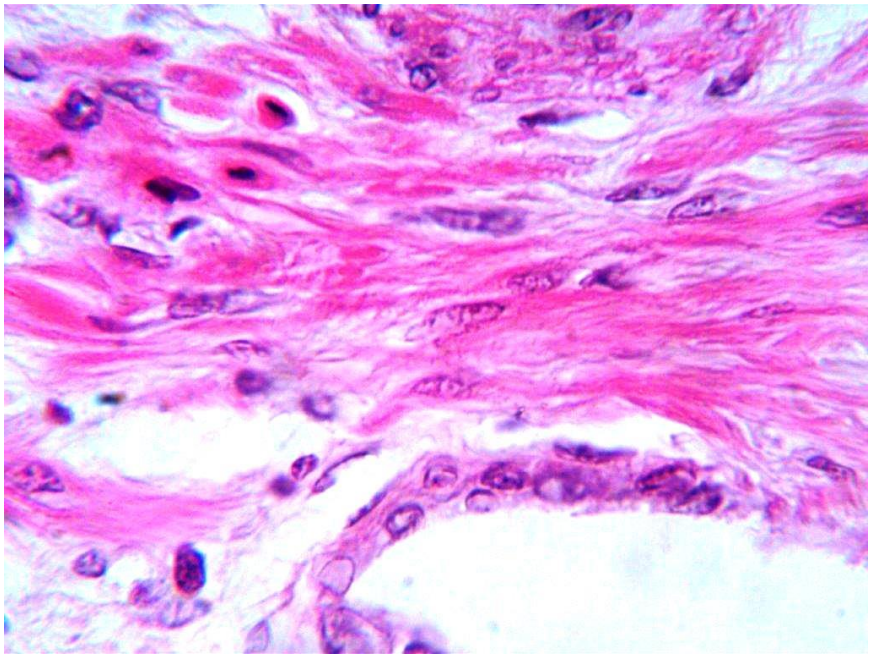
Other putative cancers are also discussed.

4 THE ECM

The extra cellular matrix, ECM, is a complex of proteins which occupy the space outside of the cell and provide a "structure" to the cellular complex. In contrast to the TME, the ECM is an amalgam of supporting elements that create in conjunction with cells a stable homeostatic element in the human body. However, the ECM like the TME can be hijacked by the cancer cells and Thus, it is essential to understand its functioning.

4.1 OVERVIEW

We start with some recent work on the ECM. The image below is from a prostate slide and the gland is at the bottom and the ECM fibers are above.



From Kumar et al we have:

The ECM is a network of interstitial proteins that constitutes a significant proportion of any tissue.

Cell interactions with ECM are critical for development and healing, as well as for maintaining normal tissue architecture. Much more than a simple "space filler" around cells, ECM serves several key functions:

1. *Mechanical support for cell anchorage and cell migration, and maintenance of cell polarity.*
2. *Control of cell proliferation, by binding and displaying growth factors and by signaling through cellular receptors of the integrin family. The ECM provides a depot for a variety of latent growth factors that can be activated within a focus of injury or inflammation.*

3. *Scaffolding for tissue renewal. Because maintenance of normal tissue structure requires a basement membrane or stromal scaffold, the integrity of the basement membrane or the stroma of parenchymal cells is critical for the organized regeneration of tissues. Thus, ECM disruption results in defective tissue regeneration and repair, for example, cirrhosis of the liver resulting from the collapse of the hepatic stroma in various forms of hepatitis.*
4. *Establishment of tissue microenvironments. The basement membrane acts as a boundary between the epithelium and underlying connective tissue; it does not just provide support to the epithelium but is also functional, for example, in the kidney, forming part of the filtration apparatus.*

Cell surface integrins interact with the cytoskeleton at focal adhesion complexes (protein aggregates that include vinculin, α-actinin, and talin. This can initiate the production of intracellular messengers or can directly transduce signals to the nucleus. Cell surface receptors for growth factors can activate signal transduction pathways that overlap with those mediated through integrins. Signals from ECM components and growth factors can be integrated by the cells to produce a given response, including changes in proliferation, locomotion, and/or differentiation.

The ECM is constantly being remodeled; its synthesis and degradation accompany morphogenesis, tissue regeneration and repair, chronic fibrosis, and tumor invasion and metastasis. ECM occurs in two basic forms: interstitial matrix and basement membrane

Interstitial matrix is present in the spaces between cells in connective tissue, and between the parenchymal epithelium and the underlying supportive vascular and smooth muscle structures. The interstitial matrix is synthesized by mesenchymal cells (e.g., fibroblasts), forming an amorphous three-dimensional gel. Its major constituents are fibrillar and nonfibrillar collagens, as well as fibronectin, elastin, proteoglycans, hyaluronate, and other constituents.

Basement membrane. The seemingly random array of interstitial matrix in connective tissues becomes highly organized around epithelial cells, endothelial cells, and smooth muscle cells, forming the specialized basement membrane. This is synthesized conjointly by the overlying epithelium and the underlying mesenchymal cells, forming a flat lamellar "chicken wire" mesh (although labeled as a membrane, it is quite porous). The major constituents are amorphous nonfibrillar type IV collagen and laminin.

The components of the ECM fall into three groups of proteins:

1. *Fibrous structural proteins such as collagens and elastins that confer tensile strength and recoil*

2. *Water-hydrated gels such as proteoglycans and hyaluronan that permit compressive resistance and lubrication*

3. *Adhesive glycoproteins that connect ECM elements to one another and to cells*

As Liu et al note, the ECM has that "soil" like quality:

Tumor cells reside in a highly complex and heterogeneous tumor microenvironment (TME), which is composed of a myriad of genetically stable non-cancer cells, including fibroblasts, immune cells, endothelial cells, and epithelial cells, and a tumor-specific extracellular matrix (ECM).

Cancer-associated fibroblasts (CAFs), as an abundant and active stromal cell population in the TME, function as the signaling center and remodeling machine to aid the creation of a desmoplastic tumor niche. Although there is no denial that the TME and CAFs may have anti-tumor effects as well, a great deal of findings reported in recent years have convincingly revealed the tumor-promoting effects of CAFs and CAF-derived ECM proteins, enzymes, chemical factors and other downstream effectors.

While there is growing enthusiasm for the development of CAF-targeting therapies, a better understanding of the complexities of CAF-ECM and CAF-cancer cell interactions is necessary before novel therapeutic strategies ***targeting the malignant tumor "soil" can be successfully implemented in the clinic.***

4.2 Extra Cellular Matrix

The focus on intracellular pathways has been a prime direction of research in the development of cancers. However, there has from time to time been some focus on the extracellular matrix, the "ECM", which relates in many ways to the stability of the cell, its localization. Cancer cells lose this sense of localization and begin to move.

The processes at play in the ECM have a significant impact on the processes that occur within a cell. Thus, it is essential to have an understanding of the ECM. Recent work by Fisher and his people on MDA-9, a controller of certain ECM elements, demonstrates a control path that influences the internal pathways. We discuss the ECM in the context of the MDA-9 developments.

In this section we use a recent development in understanding the impact of Mda-9 and the nexus with the extra cellular matrix, ECM, and the control of metastatic melanoma.

We first review the Fisher Team efforts as recently presented and then we examine the standard intracellular pathways that have been examined and from that we provide an overview of the extra cellular matrix, ECM, which is the "glue" binding together cells and facilitating cell to cell communications.

We find this an interesting focus or research for several reasons:

1. It examines the ECM which has received limited focus.

2. It focuses on pathways as we have been also doing and specifically an interesting adjunct to the current B-RAF approach.

3. It establishes a clear path forward which is logically and experimentally based and verifiable.

There has been limited prior research on these issues. In Hearing and Leong, 380-386, there is a limited discussion regarding the ECM and melanoma with references. The work by Zent and Pozzi provides a broad and detailed perspective of the ECM with many cancers. However, their work is not specific to melanoma. In Weinberg there are references but there does not appear to be any singular focus on the ECM as a standalone system element.

4.3 Recent Evidence

In a recent paper by Das et al, the authors (from Fisher's Lab at Virginia Commonwealth) state[5]:

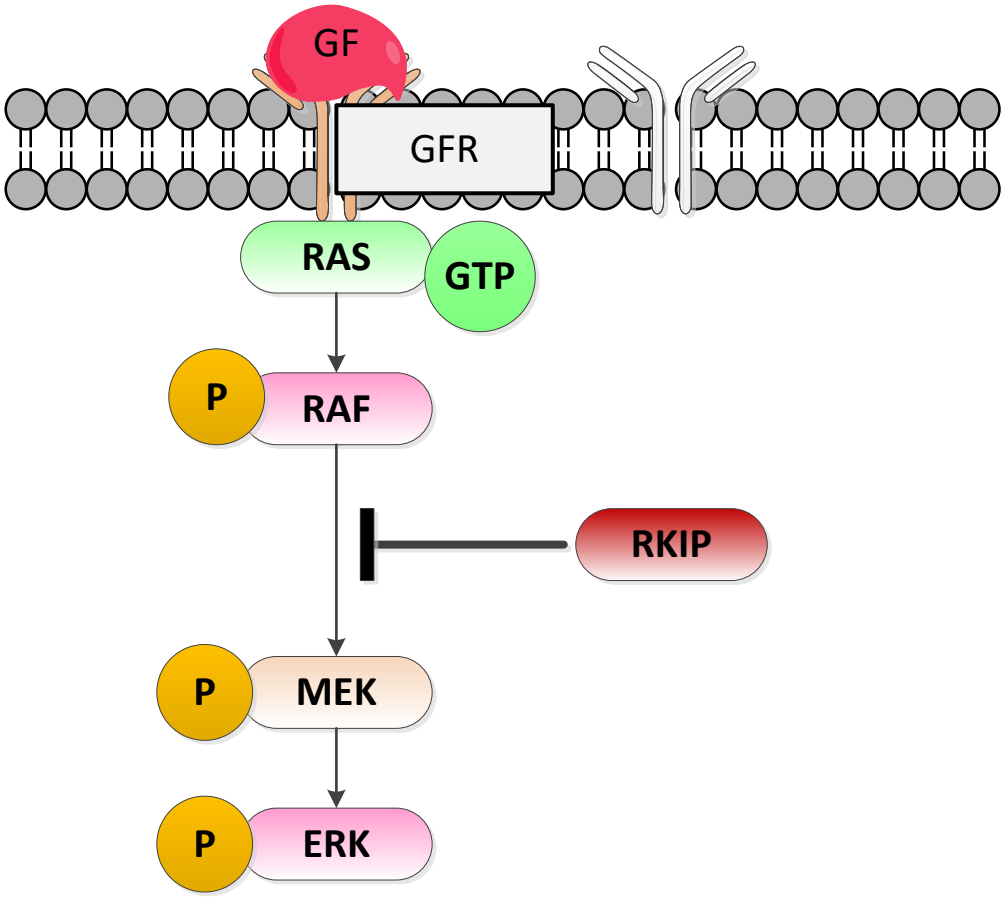
Melanoma differentiation associated gene-9 (MDA-9), also known as syntenin, functions as a positive regulator of melanoma progression and metastasis. In contrast, the Raf kinase inhibitor RKIP, a negative modulator of RAF-stimulated MEKK activation, is strongly downregulated in metastatic melanoma cells. In this study, we explored an hypothesized inverse relationship between MDA-9 and RKIP in melanoma. Tumor array and cell line analyses confirmed an inverse relationship between expression of MDA-9 and RKIP during melanoma progression.

We found that MDA-9 transcriptionally downregulated RKIP in support of a suggested crosstalk between these two proteins. Further, MDA-9 and RKIP physically interacted in a manner that correlated with a suppression of FAK and c-Src phosphorylation, crucial steps necessary for MDA-9 to promote FAK/c-Src complex formation and initiate signaling cascades that drive the MDA-9-mediated metastatic phenotype.

Lastly, ectopic RKIP expression in melanoma cells overrode MDA-9-mediated signaling, inhibiting cell invasion, anchorage-independent growth and in vivo dissemination of tumor cells. Taken together, these findings establish RKIP as an inhibitor of MDA-9-dependent melanoma metastasis, with potential implications for targeting this process therapeutically.

From the paper by Houben et al we have the RKIP activation as shown below:

[5] http://cancerres.aacrjournals.org/search?author1=Swadesh+K+Das&sortspec=date&submit=Submit ; Therapeutics, Targets, and Chemical Biology Raf Kinase Inhibitor RKIP Inhibits MDA-9/Syntenin-Mediated Metastasis in Melanoma, Das, S., et al, *Cancer Res Published Online First October 11, 2012.*



As Houben et al state:

The Ras/Raf/MEK/ERK intracellular signalling cascade is a major determinant in the control of cell growth, differentiation, and survival and can be activated in response to a variety of extracellular stimuli. Stimulation of growth factor receptors results in the activation of the small G-protein Ras, which in turn interacts with the protein kinase Raf leading to its activation. MAP kinase kinase kinase (Raf) phosphorylates and activates MAP kinase kinase (MEK), and MEK phosphorylates and activates extracellular signal-regulated kinase (ERK) 1/2 (p42/p44 MAP kinases).

Although Raf and MEK appear largely restricted to only one class of substrates, ERK targets more than 70 substrates including membrane, cytoskeletal, cytoplasmic, nuclear, and even mitochondrial proteins. Recently, a negative regulator of this pathway has been described. The Raf Kinase Inhibitor Protein (RKIP) binds to either Raf or MEK and thereby interferes with the activation of MEK by Raf. The importance of the Ras/Raf/MEK/ERK signalling pathway for carcinogenesis is well established. Indeed, Ras genes (K-ras, H-ras, and N-ras) are the most frequently mutated oncogenes detected in human cancer.

Houben et al further state about RKIP (12q24.23) as a target the following:

To assess the relevance of the Ras/Raf/MEK/MAP kinase pathway, we analyzed for activating B-Raf mutations and we elucidated the presence of the Raf Kinase Inhibitor Protein (RKIP) and extracellular signal-regulated kinase (ERK) as well as the phosphorylation status of ERK. All MCC samples were negative for the B-Raf$_{V600E}$ mutation. Remarkably, RKIP, which was shown to interfere with the activation of MEK by Raf, was highly expressed in primary as well as in metastatic MCC. … Western blot analysis of three MCC-derived cell lines revealed in one case the pattern present in situ (i.e. high RKIP expression and complete absence of phosphorylated ERK).

Thus, the Fisher team seems to seek out a RKIP inhibitor to slow the pathway. This is in addition to the B-RAF inhibitors which are currently in clinical use.

Now in an industry piece on the same article the author Ho states[6]:

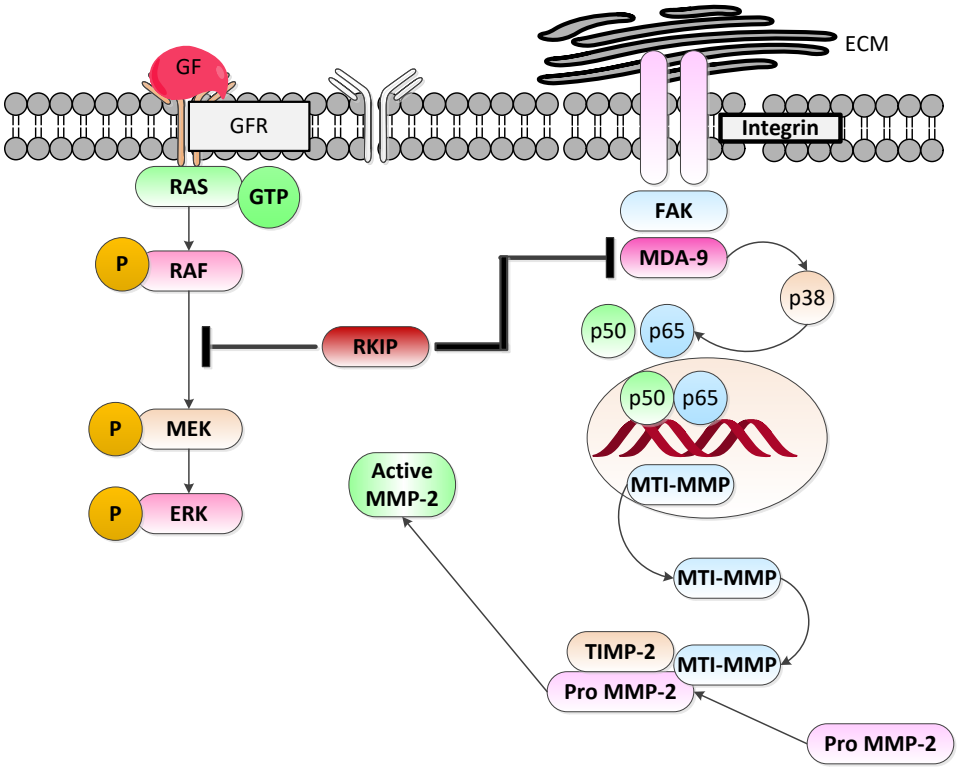
…. the scientist believes that they have the ability to eliminate melanoma differentiation associated gene-9 (mda-9)/syntenin, a specific protein. In the experiment, the researchers discovered that Raf kinase inhibitor protein (RKIP) was able to interact and suppress with mda-9/syntenin. The protein was originally cloned in a laboratory and past studies showed how it interacted with c-Src, another protein, to produce a set of chemical reactions that later boosted metastasis.

"Prior research suggests that RKIP plays a seminal role in inhibiting cancer metastasis, but, until now, the mechanisms underlying this activity were not clear," explained Paul Fisher, the program co-leader of Cancer Molecular Genetics at Virginia Commonwealth University Massey Cancer Center, in a prepared statement. "In addition to providing a new target for future therapies, there is potential for using these two genes as biomarkers for monitoring melanoma development and progression."

The team of investigators discovered that RKIP become attached to mda-9/syntenin, which resulted in limiting the expression of mda-9/syntenin. With the finding of this physical interaction, the scientists believe that they could possibly create small molecules that are similar to RKIP and the molecules could be used as drugs to treated metastasis in cancers like melanoma.

We depict this pathway below:

[6] http://www.redorbit.com/news/health/1112732493/stopping-the-spread-of-melanoma-by-removing-protein-affecting-metastasis/ ; Ho, C., Stopping The Spread Of Melanoma By Removing Protein Affecting Metastasis, RedOrbit, November 15, 2012



The article continues:

There was also a difference in terms of the level of mda-9/syntenin and RKIP. While malignant and metastasis melanoma cells had higher levels of mda-9/sytnenin compared to RKIP, the healthy melanocyte cells that create pigment in eyes, hair, and skin had higher levels of RKIP than mda-9/syntenin. The researchers believe that different levels in the proteins could be used in diagnosis, particularly in following the progression of a disease or tracking a patient's response to a particular treatment.

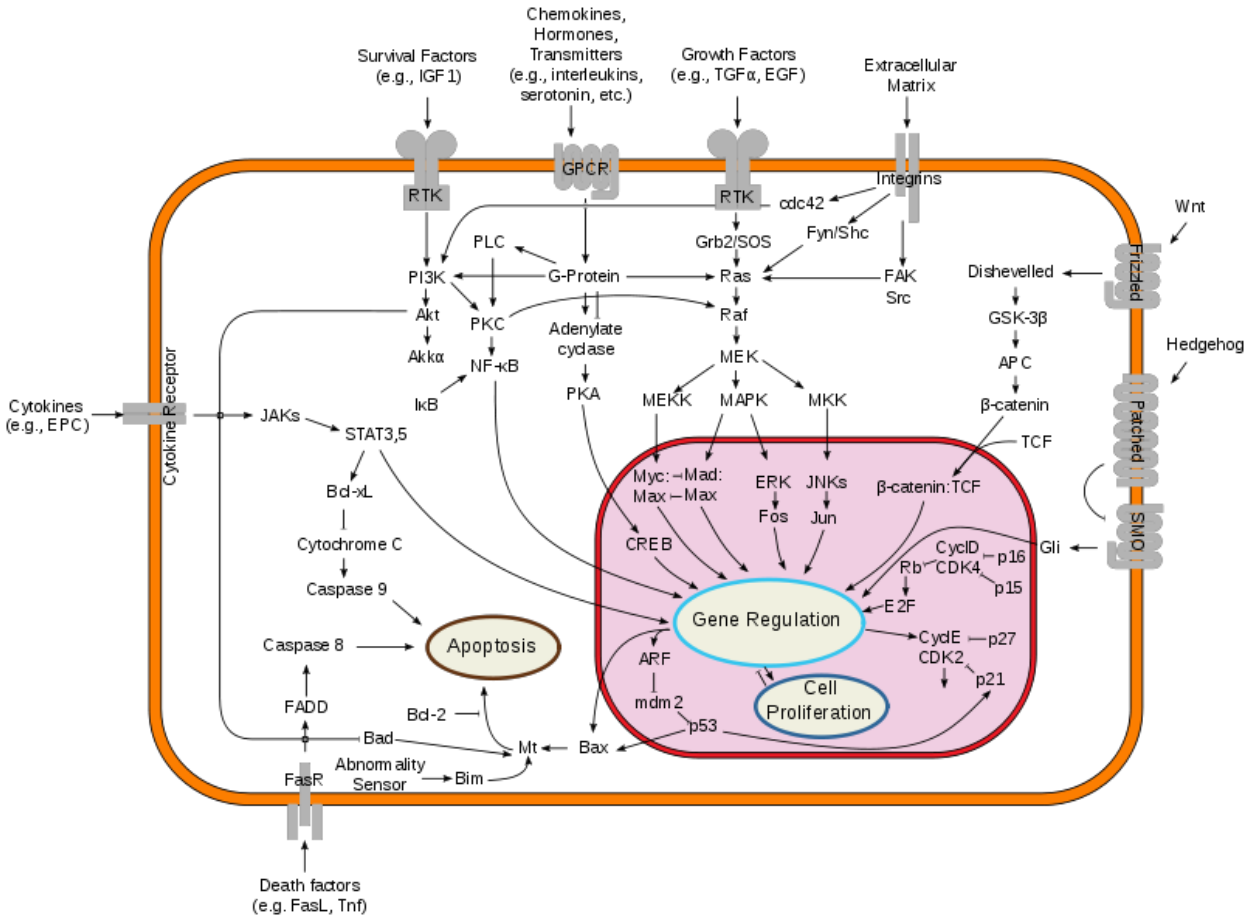
"Our findings represent a major breakthrough in understanding the genetic mechanisms that lead to metastasis in melanoma. Prior studies have shown that levels of mda-9/syntenin are elevated in a majority of cancers, including melanoma, suggesting that our findings could be applicable for a wide range of diseases,"

Moving forward, the scientists plan to determine how they can develop small molecules that mimic RKIP. These molecules could potentially be utilized in new treatments for melanoma.

This is a fundamental result. It demonstrates another pathway element and at the same time connects the intracellular pathways with the extra cellular matrix and their pathways. Potentially this is diagnostic, prognostic and a treatment as well.

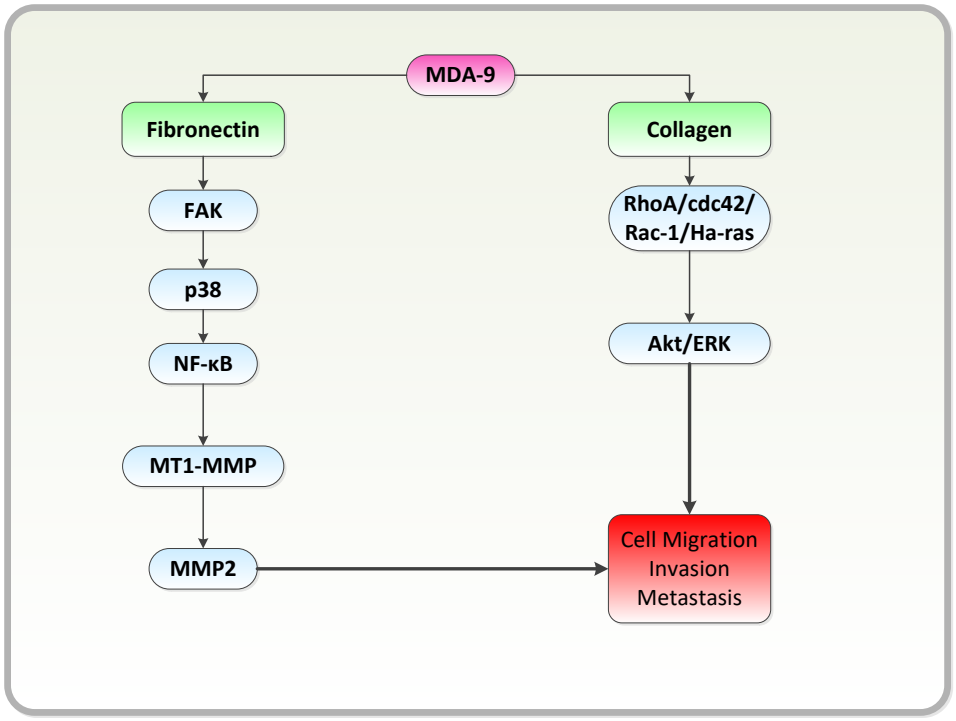
4.4 STANDARD INTRA-CELLULAR PATHWAYS

The following Figure is a repetition of the standard intra-cellular pathways. We have discussed these at length.



What is different from what we have detailed previously is the Extra Cellular Matrix connection via the integrins. This yields the controlling FAK path using FAK and Src. Note that this activates RTK and Ras and Thus, as we have described many of the other internal pathways this is the first time we have involved the ECM directly. The ECM is a significant element in cancer proliferation, it is the sea in which the changing cells sail metaphorically but at the same time it allows communication with the environment as well as presenting ligands to receptors.

As depicted in Sarkar et al, we have the following sets of paths and the results:



We shall be examining these in some detail. Let us first characterize some of the above identified elements controlled by the extracellular matrix path. The others we have examined in detail elsewhere.

4.4.1 FAK

FAK is also known as; PTK2, FADK; FAK1; FRNK; PPP1R71; p125FAK; pp125FAK. It is located at 8q24.3. It is a kinase.

NCBI states its function as follows:

This gene encodes a cytoplasmic protein tyrosine kinase which is found concentrated in the focal adhesions that form between cells growing in the presence of extracellular matrix constituents. The encoded protein is a member of the FAK subfamily of protein tyrosine kinases but lacks significant sequence similarity to kinases from other subfamilies. Activation of this gene may be an important early step in cell growth and intracellular signal transduction pathways triggered in response to certain neural peptides or to cell interactions with the extracellular matrix. Several transcript variants encoding different isoforms have been found for this gene, but the full-length natures of only three of them have been determined.

4.4.2 Src

SRC is located at 20q12-q13. As noted in NCBI[7]:

[7] http://www.ncbi.nlm.nih.gov/gene/6714

This gene is highly similar to the v-src gene of Rous sarcoma virus. This proto-oncogene may play a role in the regulation of embryonic development and cell growth. The protein encoded by this gene is a tyrosine-protein kinase whose activity can be inhibited by phosphorylation by c-SRC kinase. Mutations in this gene could be involved in the malignant progression of colon cancer. Two transcript variants encoding the same protein have been found for this gene.

4.4.3 p38

The p38 gene has multiple names. It is MAPK14, RK; CSBP; EXIP; Mxi2; CSBP1; CSBP2; CSPB1; PRKM14; PRKM15; SAPK2A; p38ALPHA. It is located at 6p21.3-p21.2.

Its function described by NCBI is as follows[8]:

The protein encoded by this gene is a member of the MAP kinase family. MAP kinases act as an integration point for multiple biochemical signals, and are involved in a wide variety of cellular processes such as proliferation, differentiation, transcription regulation and development.

This kinase is activated by various environmental stresses and proinflammatory cytokines.

The activation requires its phosphorylation by MAP kinase kinases (MKKs), or its autophosphorylation triggered by the interaction of MAP3K7IP1/TAB1 protein with this kinase. The substrates of this kinase include transcription regulator ATF2, MEF2C, and MAX, cell cycle regulator CDC25B, and tumor suppressor p53, which suggest the roles of this kinase in stress related transcription and cell cycle regulation, as well as in genotoxic stress response.

Four alternatively spliced transcript variants of this gene encoding distinct isoforms have been reported.

4.4.4 NF-κB

We have discussed this before. We reiterate what that discussion contains.

NF-κB is a transcription factor that resides in the cytoplasm. It is called Nuclear Factor and was identified by David Baltimore as an enhancer factor for the κ chain of Ig light chain in B lymphocytes. When activated it moves to the nucleus and is a transcription factor in activating over 400 genes. It is activated by a large number of stimuli and its action of a large gene set causes significant DNA activity. NF-κB appears on 10q24 and is somatic and acts in a dominant manner.

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

[8] http://www.ncbi.nlm.nih.gov/gene/1432

transcript and protein suggesting that a marked increase in AR expression is a critical event in therapy resistance…

Recent studies also demonstrate that increased AR expression is both necessary and sufficient to convert prostate cancer growth from a hormone therapy-sensitive to a resistant state in xenograft models...Since AR mRNA levels are often increased in ADRPC without gene amplification , …

it is likely mediated by transcription factors and transcription regulating signal transduction pathways that are altered during progression.

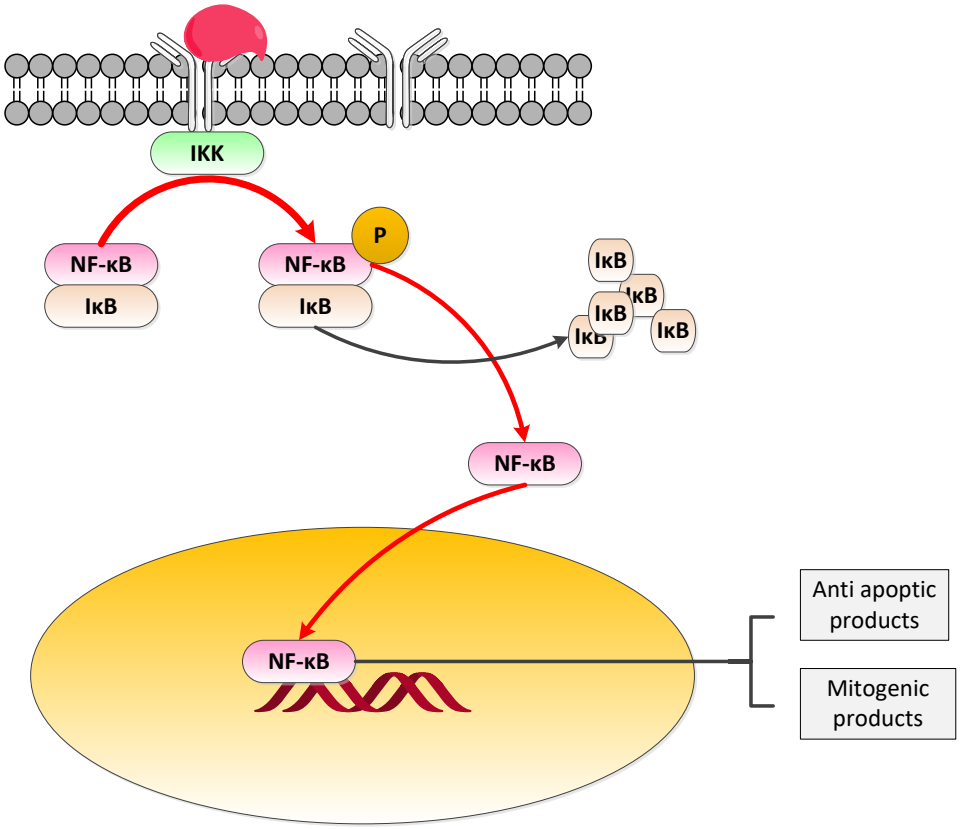
Nuclear Factor (NF)-κB is a family of transcription factors composed of homo- and hetero-dimers initially identified as an enhancer binding protein for the immunoglobulin light chain in B lymphocytes…

Zhang continues:

Several studies have examined the expression of NF-κB in human prostate cancer and its relationship to clinical features of the disease. NF-κB/p65 is overexpressed in prostatic intraepithelial neoplasia and cancer compared with benign epithelium. Nuclear levels of NF-κB/p65 correlate with NF-κB-dependent expression of BclII, cyclin D1, matrix metalloproteinase-9, and vascular endothelial growth factor.

Recent work indicates that NF-κB/p65 expression is predictive of biochemical recurrence in patients with positive surgical margins after radical prostatectomy and nuclear localization of NF-κB is increased in prostate cancer lymph node metastasis and can be used to predict patient outcome. These results demonstrate that NF-κB/p65 is frequently activated in human prostate adenocarcinoma and expression may be related to progression.

We now depict this putative pathway based upon the work of Kwang and Aggarwal. This is shown below. Activated NF-κB is clearly an activator of an anti-apoptosis process in the nucleus. The paper by Huang et al shows that blockade of NF-κB is an effective suppressor of angiogenesis, invasion and metastasis of prostate cancer.



NF-κB is another transcription protein seen in melanoma. This protein is characterized by:

- NF-κB is a transcription factor that resides in the cytoplasm.
- It is called Nuclear Factor and was identified by David Baltimore as an enhancer factor for the κ chain of Ig light chain in B lymphocytes
- When activated it moves to the nucleus and is a transcription factor in activating over 400 genes
- It is activated by a large number of stimuli and its action of a large gene set causes significant DNA activity
- NF-κB appears on 10q24 and is somatic and acts in a dominant manner.

NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) is a protein complex that controls the transcription of DNA. NF-κB is widely used by eukaryotic cells as a regulator of genes that control cell proliferation and cell survival. As such, many different types of human tumors have mis-regulated NF-κB: that is, NF-κB is constitutively active. Active NF-κB turns on the expression of genes that keep the cell proliferating and protect the cell from conditions that would otherwise cause it to die via apoptosis.

As Amiri and Richmond state:

Nuclear Factor-kappa B (NF-κB) is an inducible transcription factor that regulates the expression of many genes involved in the immune response. Recently, NF-κB activity has been shown to be upregulated in many cancers, including melanoma. Data indicate that the enhanced

activation of NF- κB may be due to deregulations in upstream signaling pathways such as Ras/Raf, PI3K/Akt, and NIK. Multiple studies have shown that NF-κB is involved in the regulation of apoptosis, angiogenesis, and tumor cell invasion, all of which indicate the important role of NF-κB in tumorigenesis. Thus, understanding the molecular mechanism of melanoma progression will aid in designing new therapeutic approaches for melanoma.

They continue:

Constitutive activation of NF-κB is an emerging hallmark of various types of tumors including breast, colon, pancreatic, ovarian, and melanoma. In the healthy human, NF-κB regulates the expression of genes involved in normal immunologic reactions (e.g. generation of immunoregulatory molecules such as antibody light chains) in response to proinflammatory cytokines and by-products of microbial and viral infections. NF-κB also modulates the expression of factors responsible for growth as well as apoptosis. However, increased activation of NF-κB results in enhanced expression of proinflammatory mediators, leading to acute inflammatory injury to lungs and other organs, and development of multiple organ dysfunctions as well as cancer.

They then summarize NF-kB's role in melanoma as:

3.1. Apoptosis resistance and cell proliferation*: In processes such as tumor initiation and promotion where prolonged survival of cells is a crucial event, NF-κB plays an important role as a mediator of inhibition of apoptosis. In melanoma, NF-κB has been shown to activate expression of anti-apoptotic proteins such as tumor necrosis factor receptor-associated factor 1 (TRAF1), TRAF2, and the inhibitor-of apoptosis (IAP) proteins c-IAP1, c-IAP2, and melanoma inhibitor of apoptosis (ML-IAP), survivin as well as Bcl-2 like proteins…*

3.2. Invasion and metastasis*: In invasion and metastasis of melanoma, NF-κB may regulate the production of prostaglandins via cyclooxygenase-2 (COX-2), which has been shown to be overexpressed in melanoma. It was shown that COX-2 is expressed in the majority of primary malignant melanoma, as well as in five human malignant melanoma cell lines….*

However, as Liu et al (2006) state:

Malignant melanoma is the most lethal skin cancer, whose ability to rapidly metastasize often prevents surgical cure.

Furthermore, the systemic treatment of melanoma is largely ineffective due to the intrinsic resistance of melanoma cells to numerous anticancer agents. Increased survival of melanoma cells is primarily attributed to the constitutive activation of the transcription factor nuclear factor kB (NF-kB), which regulates the expression of many anti-apoptotic, pro-proliferative and pro-metastatic genes.

Canonical activation of the NF-kB pathway occurs when NF-kB switches its localization from the cytoplasm, where it is maintained inactive by assembly with the inhibitor IkB protein, to the nucleus, where NF-kB regulates gene expression. NF-kB activation relies upon the

phosphorylation dependent ubiquitination and degradation of IkB mediated by the IkB kinase (IKK) complex and b-Trcp E3 ubiquitin ligases.

Consequently, both IKK activity and the levels of b-Trcp regulate the extent of IkB degradation and hence NF-kB activation. The genetic basis that underlies the elevated NF-kB activity in malignant melanoma largely remains elusive.

Constitutively active IKK has been demonstrated to sustain NF-kB activation in human melanoma cells, resulting in induction of the chemokine CXCL1. CXCL1, in turn, is capable of activating IKK and NF-kB and promoting cell survival and tumorigenesis …. However, the original genetic alterations that initiate this feed-forward mechanism in melanoma remain unclear.

One of the major oncogenic events described in the genesis of malignant melanoma is constitutive activation of the Ras-regulated RAF-MEK-ERK mitogen-activated protein kinase (MAPK) pathway. This is achieved most frequently by activating mutations in either BRAF (e.g. V600E substitution) or, less frequently, in N-RAS … Recent evidence indicates that oncogenic BRAF activity is essential for human melanoma cell growth and survival …

However, despite prior reports that RAF can activate NF-kB …, the mechanism(s) by which $BRAF_{V600E}$ ($BRAF_{VE}$) may elicit NF-kB signaling in melanoma cells have not yet been elucidated. Activation of the canonical NF-kB pathway depends on both IKK activity, which has been shown to be elevated in human melanomas….

Liu et al conclusion is speculative but telling:

Taken together, these data support a model in which mutational activation of BRAF in human melanomas contributes to constitutive induction of NF-κB activity and to increased survival of melanoma cells.

Again, we have the issue of speculation as to where and why the mutations occur. Here they speculate about the BRAF mutation resulting in the antiapoptotic control with NF-κB.

4.4.5 MMP-9

As NCBI states[9]:

Proteins of the matrix metalloproteinase (MMP) family are involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling, as well as in disease processes, such as arthritis and metastasis. Most MMP's are secreted as inactive proproteins which are activated when cleaved by extracellular proteinases.

[9] http://www.ncbi.nlm.nih.gov/gene/4318

The enzyme encoded by this gene degrades type IV and V collagens. Studies in rhesus monkeys suggest that the enzyme is involved in IL-8-induced mobilization of hematopoietic progenitor cells from bone marrow, and murine studies suggest a role in tumor-associated tissue remodeling

4.4.6 CDC42

CDC42 also plays a significant role. As NCBI states[10]:

The protein encoded by this gene is a small GTPase of the Rho-subfamily, which regulates signaling pathways that control diverse cellular functions including cell morphology, migration, endocytosis and cell cycle progression. This protein is highly similar to Saccharomyces cerevisiae Cdc 42, and is able to complement the yeast cdc42-1 mutant.

The product of oncogene Dbl was reported to specifically catalyze the dissociation of GDP from this protein. This protein could regulate actin polymerization through its direct binding to Neural Wiskott-Aldrich syndrome protein (N-WASP), which subsequently activates Arp2/3 complex. Alternative splicing of this gene results in multiple transcript variants.

4.5 THE EXTRACELLULAR MATRIX

The ECM has often been neglected when discussing cancer pathways. Weinberg has multiple references but does not seem to place it in any specific spotlight. In Lewin, Cell, the discussion is quite well focused but yet there is but passing reference to the impact on cancer pathways. Specifically, there is reference to MMP-9[11], here a metalloproteinase, and melanoma[12].

The ECM is the collection of molecules that lie between the cell walls. The ECM provides for structural integrity as well as facilitates and even participates in cell to cell communications. The ECM is a highly complex and quite active element in the ongoing life of the cells. In addition we all too often look to what happens in a cell, with at best a nod to ligands, and we do not look at the cell internals as well as the ECM as a holistic system totality. The work of the Fisher Team in a small way may help refocus this effort on the complex as a working whole.

We will deal with the principal participants in the ECM. There are a wealth of books which focus on this area.

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[10] http://www.ncbi.nlm.nih.gov/gene/998

[11] See Lewin (p 850)

[12] As NCBI states: "*Proteins of the matrix metalloproteinase (MMP) family are involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling, as well as in disease processes, such as arthritis and metastasis. Most MMP's are secreted as inactive proproteins which are activated when cleaved by extracellular proteinases. The enzyme encoded by this gene degrades type IV and V collagens. Studies in rhesus monkeys suggest that the enzyme is involved in IL-8-induced mobilization of hematopoietic progenitor cells from bone marrow, and murine studies suggest a role in tumor-associated tissue remodeling.*" see http://www.ncbi.nlm.nih.gov/gene/4318

4.5.1 Collagen

Collagens provide structure support. They are triple helical proteins wrapped to provide that supporting structure between the cells. There any many types of collagen and the actually assembly commences within the cell and the semi-finished product passes through the cell wall to the ECM. For our purposes the collagen complexes are at this time of limited interest. From Kumar et al we also have:

Collagens are composed of three separate polypeptide chains braided into a ropelike triple helix. About 30 collagen types have been identified, some of which are unique to specific cells and tissues. Some collagen types (e.g., types I, II, III, and V collagens) form linear fibrils stabilized by interchain hydrogen bonding; such fibrillar collagens form a major proportion of the connective tissue in structures such as bone, tendon, cartilage, blood vessels, and skin, as well as in healing wounds and scars.

The tensile strength of the fibrillar collagens derives from lateral crosslinking of the triple helices by covalent bonds, an unusual post-translational modification that requires hydroxylation of lysine residues in collagen by the enzyme lysyl oxidase. Because lysyl oxidase is a vitamin C-dependent enzyme, children with ascorbate deficiency have skeletal deformities, and people of any age with vitamin C deficiency heal poorly and bleed easily because of "weak" collagen. Genetic defects in collagens cause diseases such as osteogenesis imperfecta and certain forms of Ehlers-Danlos syndrome.

Nonfibrillar collagens variously contribute to the structures of planar basement membranes (type IV collagen); help regulate collagen fibril diameters or collagen-collagen interactions via so-called "fibril-associated collagen with interrupted triple helices" (FACITs, such as type IX collagen in cartilage); and provide anchoring fibrils within basement membrane beneath stratified squamous epithelium (type VII collagen).

4.5.2 Elastin.

From Kumar et al we have the following discussion on elastin:

The ability of tissues to recoil and recover their shape after physical deformation is conferred by elastin. Elasticity is especially important in cardiac valves and large blood vessels, which must accommodate recurrent pulsatile flow, as well as in the uterus, skin, and ligaments. Morphologically, elastic fibers consist of a central core of elastin with an associated meshlike network composed of fibrillin. The latter relationship partially explains why fibrillin defects lead to skeletal abnormalities and weakened aortic walls, as in individuals with Marfan syndrome. Fibrillin also controls the availability of TGF-β.

4.5.3 Fibronectin

Fibronectin facilitates the process of connecting cells to matrices of collagen. Fibronectin proteins have a six element structure. Cells bind to fibronectin via receptors called integrins. The fibronectin binding Thus, activates pathways within the cell, thereby establishing an intra and intercellular pathway complex. The pathways activated control growth, movement and cell differentiation.

We can now examine some of the relevant literature on fibronectin and melanomas. As Yi and Ruoslahti state:

Fibronectin is a prototypic extracellular matrix (ECM) protein that is deposited by various types of cells into an adhesive fibrillar meshwork of protein.

Fibronectin, and ECM in general, control many cellular functions, including growth, migration, differentiation, and survival.

The signals that control these behaviors are transmitted from the ECM to the cell by integrins, a family of transmembrane receptors. Malignant cells often bypass the ECM–integrin signaling system; they are not bound by the spatial constraints imposed by the ECM on normal cells, and they no longer require ECM contact for survival

Namely fibronectin is a broad-based controller of many cellular processes. Understanding them may open options for therapeutics. Liu et al state:

Tumor cells frequently exhibit decreased adhesiveness due to failure to deposit stromal fibronectin (FN), permitting more rapid proliferation, migration, invasion, and metastasis. Although up-regulation of FN has been noted in gene profiles of carcinomas compared with normal tissue, reduced FN expression has been described at the peripheral margins of invading tumors. In this study, we investigate the role of FN in cancer behavior. …

Loss of spatial stability is a common feature of many malignancies. Cells proliferate and the loss of structure characteristic result in disoriented masses of the new cells as they multiply.

Neoplastic transformation is often characterized by changes in the organization of the cytoskeleton, decreased cell adhesion, and aberrant adhesion–mediated signaling. Disruption of normal cell adhesion contributes to enhanced proliferation, migration, and invasion leading to metastasis. Fibronectin (FN) is an extracellular matrix protein with putative roles in mediating these actions. Indeed, tumor cells with decreased adhesiveness frequently fail to deposit stromal FN.

In particular, reduced FN expression has been noted in transformed cell lines and primary tumors, including thyroid cancer, where diminished FN has been identified at the periphery of invasive tumor margins. In this context, we found that down-regulation of FN stimulates thyroid cancer cell proliferation and tumor growth.

Conversely, 1, 25-dihydroxy vitamin D3 treatment increases cell adhesiveness and inhibits cell proliferation and tumor growth through enhanced FN expression.

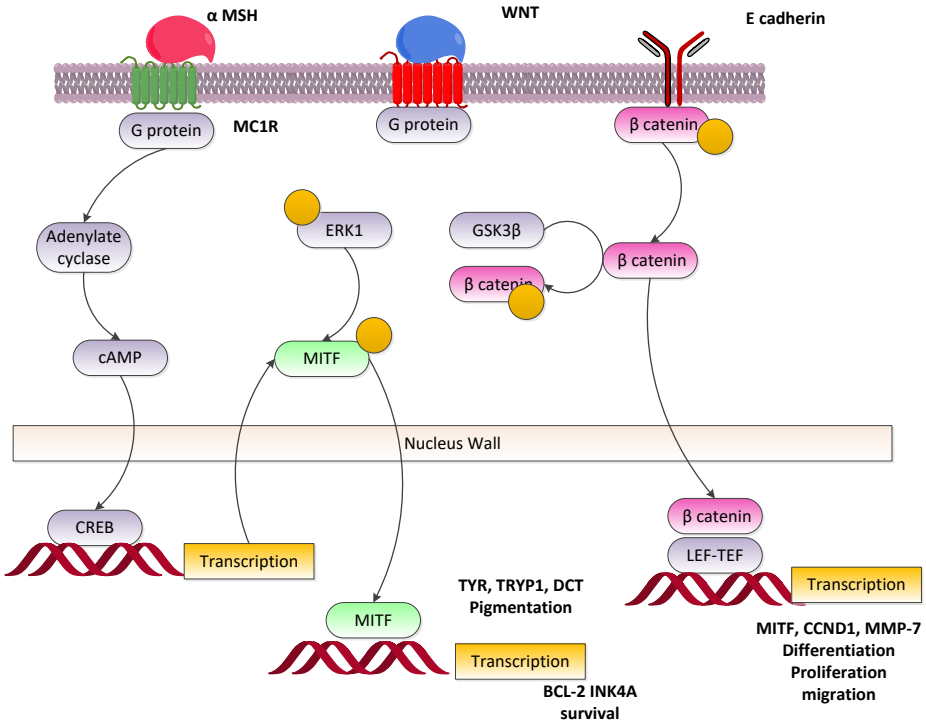
From Kumar et al we also have:

This is a large (450 kD) disulfide-linked heterodimer that exists in tissue and plasma forms; it is synthesized by a variety of cells, including fibroblasts, monocytes, and endothelium. Fibronectin has specific domains that bind to distinct ECM components (e.g., collagen, fibrin, heparin, and proteoglycans), as well as integrins . In healing wounds, tissue and plasma fibronectin provide a scaffold for subsequent ECM deposition, angiogenesis, and reepithelialization.

We will come back to fibronectin in out later analysis.

4.5.4 E-cadherin

We have discussed E-cadherin at length in previous work. It plays a critical role in stabilizing cell adhesion and localization. Loss of E-cadherin results in loss of cell localization and Thus, cell movement. Specifically in melanocytes the cells begin to leave the basal layer and migrate upward as in melanoma in situ and downward as in superficial spreading melanoma.



As Swiatoniowski et al state:

Integrins are molecules which play a significant role in cell-extracellular matrix (ECM) interactions. They interact with the RGD tripeptide of fibronectin (FN), one of the main components of ECM. Labile expression of FN has been proven to play an important role both in

the normal developmental process (morphogenetic movements) and in the course of carcinogenesis …

Many authors have implicated loss or decrease of EC expression as an independent negative prognostic marker in breast cancer patients. There is increasing experimental evidence for a relationship between the EC level and different features of breast cancer, including histological grade and axillary lymph node involvement…. In conclusion, our experiment revealed no prognostic value for EC or FN expressions in a homogenous group of patients

4.5.5 Proteoglycan

Proteoglycans are single polypeptide with multiple sugars attached. They provide for hydration in the ECM. From Kumar et al we have the following details:

Proteoglycans form highly hydrated gels that confer resistance to compressive forces; in joint cartilage, proteoglycans also provide a layer of lubrication between adjacent bony surfaces. Proteoglycans consist of long polysaccharides called glycosaminoglycans (examples are keratan sulfate and chondroitin sulfate) attached to a core protein; these are then linked to a long hyaluronic acid polymer called hyaluronan in a manner reminiscent of the bristles on a test-tube brush. The highly negatively charged, densely packed sulfated sugars attract cations (mostly sodium) and abundant water molecules, producing a viscous, gelatin-like matrix. Besides providing compressibility to tissues, proteoglycans also serve as reservoirs for secreted growth factors (e.g., FGF and HGF). Some proteoglycans are integral cell membrane proteins that have roles in cell proliferation, migration, and adhesion, for example, by binding and concentrating growth factors and chemokines.

4.5.6 Protease

The proteases are ECM proteins which function to degrade the refuse in the ECM. The metalloproteinases are a family of proteases. They are also called MMP. MMP-9 and MMP-2 are ones of the MMPs often associated with melanoma.

There has been extensive work examining the MMPs and melanoma some dating back to the 1990s, see that of Luca et al. A recent result by Hoffman et al state:

Matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) are involved in tumour progression and metastasis. In this study, we investigated the in vitro and in vivo expression patterns of MMP-1, MMP-2, MMP-3, MMP-9, TIMP-1 and TIMP-2 mRNA and protein in a previously described human melanoma xenograft model.

This model consists of eight human melanoma cell lines with different metastatic behaviour after subcutaneous (s.c.) injection into nude mice. MMP-1 mRNA was detectable in all cell lines by reverse transcription polymerase chain reaction (RT-PCR), but the expression was too low to be detected by Northern blot analysis. No MMP-1 protein could be found using Western blotting. MMP-2 mRNA and protein were present in all cell lines, with the highest expression of both latent and active MMP-2 in the highest metastatic cell lines MV3 and BLM. MMP-3 mRNA was

expressed in MV3 and BLM, and in the non-metastatic cell line 530, whereas MMP-3 protein was detectable only in MV3 and BLM.

None of the melanoma cell lines expressed MMP-9. TIMP-1 and TIMP-2 mRNA and protein, finally, were present in all cell lines. A correlation between TIMP expression level and metastatic capacity of cell lines, However, was lacking. MMP and TIMP mRNA and protein expression levels were also studied in s.c. xenograft lesions derived from a selection of these cell lines.

RT-PCR analysis revealed that MMP-1 mRNA was present in MV3 and BLM xenografts, and to a lesser extent in 530. Positive staining for MMP-1 protein was found in xenograft lesions derived from both low and high metastatic cell lines, indicating an in vivo up-regulation of MMP-1. MMP-2 mRNA was detectable only in xenografts derived from the highly metastatic cell lines 1F6m, MV3 and BLM. In agreement with the in vitro results, the highest levels of both latent and activated MMP-2 protein were observed in MV3 and BLM xenografts.

With the exception of MMP-9 mRNA expression in 530 xenografts, MMP-3, MMP-9, and TIMP-1 mRNA and protein were not detectable in any xenograft, indicating a down-regulated expression of MMP-3 and TIMP-1 in vivo. TIMP-2 mRNA and protein were present in all xenografts; interestingly, the strongest immunoreactivity of tumour cells was found at the border of necrotic areas. Our study demonstrates that of all tested components of the matrix metalloproteinase system, only expression of activated MMP-2 correlates with increased malignancy in our melanoma xenograft model, corroborating an important role of MMP-2 in human melanoma invasion and metastasis.

We shall see the impact of MMPs as we examine the pathways.

4.5.7 Integrins

Integrins are for the most part the receptors for ECM proteins. They are one of many such cell surface receptors. The integrins play important roles in cell homeostasis and cell to cell communications. From Kumar et al we also have:

These are a large family of transmembrane heterodimeric glycoproteins composed of α- and β-subunits that allow cells to attach to ECM constituents such as laminin and fibronectin. Thus, functionally and structurally linking the intracellular cytoskeleton with the outside world. Integrins also mediate cell-cell adhesive interactions. For instance, integrins on the surface of leukocytes are essential in mediating firm adhesion to and transmigration across the endothelium at sites of inflammation, and they play a critical role in platelet aggregation. Integrins attach to ECM components via a tripeptide arginine-glycine-aspartic acid motif (abbreviated RGD). In addition to providing focal attachment to underlying substrates, binding through the integrin receptors can also trigger signaling cascades that influence cell locomotion, proliferation, shape, and differentiation.

4.5.8 Laminin

From Kumar et al we have:

This is the most abundant glycoprotein in the basement membrane. It is an 820-kD cross-shaped heterotrimer that connects cells to underlying ECM components such as type IV collagen and heparan sulfate. Besides mediating the attachment to the basement membrane, laminin can also modulate cell proliferation, differentiation, and motility.

4.5.9 MDA-9

Let us briefly examine the gene MDA-9 and its protein Mda-9 and what is known and how it has evolved. Now MDA-9 is located on (8q12). As the NIH data base states:

The protein encoded by this gene was initially identified as a molecule linking syndecan-mediated signaling to the cytoskeleton. The syntenin protein contains tandemly repeated PDZ domains that bind the cytoplasmic, C-terminal domains of a variety of transmembrane proteins. This protein may also affect cytoskeletal-membrane organization, cell adhesion, protein trafficking, and the activation of transcription factors.

The protein is primarily localized to membrane-associated adherens junctions and focal adhesions but is also found at the endoplasmic reticulum and nucleus. Alternative splicing results in multiple transcript variants encoding different isoforms[13].

In the paper, Src kinase activation is mandatory for MDA-9/syntenin-mediated activation of nuclear factor-κB, by H Boukerche, et al the authors state:

The scaffolding postsynaptic density-95/disks large/zonula occludens-1 (PDZ) domain-containing protein melanoma differentiation associated gene-9 (MDA-9)/syntenin is a tandem PDZ protein overexpressed in human melanoma, and breast and gastric cancer cells. MDA-9/syntenin affects cancer cell motility and invasion through distinct biochemical and signaling pathways, including focal adhesion kinase and p38 mitogen-activated protein kinase (MAPK), resulting in activation of the nuclear factor (NF)-κB pathway.

MDA-9/syntenin also promotes melanoma metastasis by activating c-Src, but how c-Src regulates NF-κB activation is unclear. Using a human melanoma model, we document that MDA-9/syntenin–c-Src interactions are positive regulators of NF-κB activation. Inhibition of c-Src by PP2 treatment, by blocking c-Src or mda-9/syntenin expression with small interfering RNA, or in c-Src (−/−) knockout cell lines, reduces NF-κB activation following overexpression of mda-9/syntenin or c-Src.

Deletion or point mutations of the PDZ binding motif preventing MDA-9/syntenin association with c-Src reveals that both PDZ domains, with PDZ2 being the dominant module, are required for activating downstream signaling pathways, including p38 MAPK and NF-κB. We also document that MDA-9/syntenin–c-Src complexes functionally cooperate with NF-κB to promote

[13] http://www.ncbi.nlm.nih.gov/gene/6386

anchorage-independent growth, motility and invasion of melanoma cells. These findings underscore PDZ domains of MDA-9/syntenin as promising potential therapeutic targets for intervening in a decisive component of cancer progression, namely, metastatic tumor spread[14]*....*

(MDA-9 Acts as a PDZ domain-containing adapter protein. In adherens junctions, it couples syndecans to cytoskeletal proteins or signaling components. Seems to be required for the targeting of TGF-alpha to the cell surface in the secretory pathway. By virtue of its association with a large number of additional proteins, including class B ephrins, TGF-alpha, phosphotyrosine phosphatase, neurofaschin, neurexin, schwannomin/merlin, IL-5 receptor, various glutamate receptor subtypes, and the syndecan family of heparan sulfate proteoglycans, MDA9 has been implicated in diverse processes, including protein trafficking, activation of the transcription factor SOX4, cytoskeleton-membrane organization, and cell adhesion/migration….

(MDA-9) Its expression is induced by IFN-gamma in melanoma cells. Is believed to be involved in cancer metastasis. In melanoma, it promotes the metastatic phenotype by activating NFkB and focal adhesion kinase (FAK), which promotes induction of matrix metalloproteinase (MMP) and then migration and extracellular matrix invasion of melanoma cells. Syntenin is overexpressed and promotes cell migration in metastatic human breast and gastric cancer cell lines.

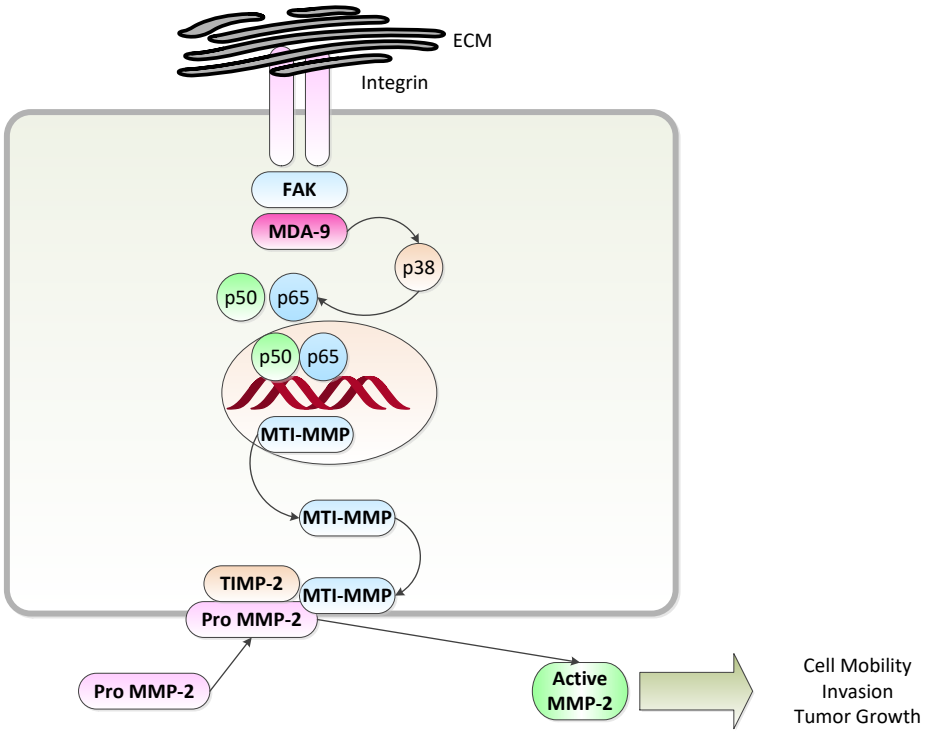
The gene product is also called by many other names, specifically:

1. MDA9
2. MDA-9
3. TGF alpha cytoplasmic domain interacting protein18
4. TACIP18
5. SYCL
6. Syntenin-1
7. Syndecan binding protein 1
8. SDCBP
9. Melanoma differentiation associated protein 9

From Das et al. we have the following modified figure[15]:

[14] http://www.nature.com/onc/journal/v29/n21/pdf/onc201065a.pdf

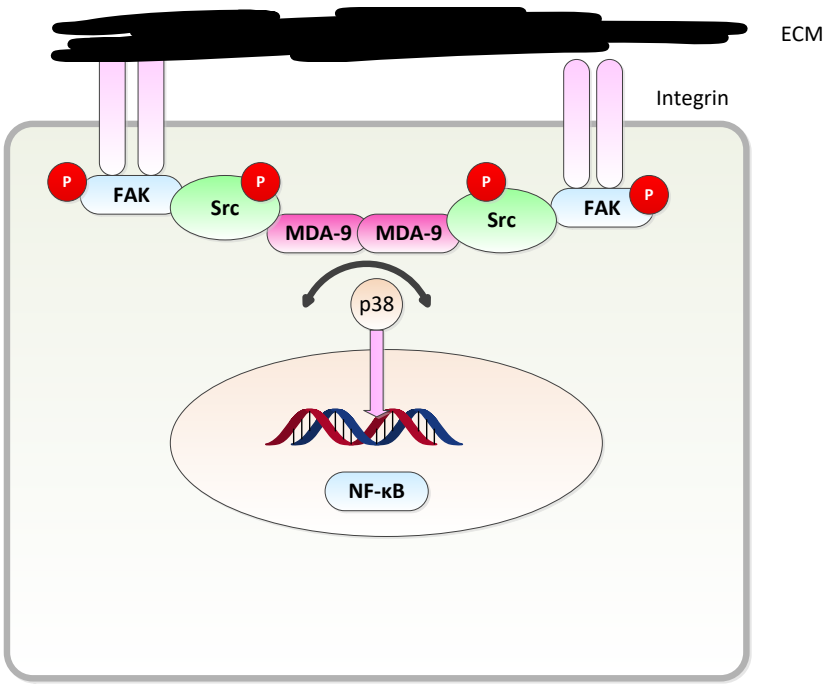
[15] http://www.bioscience.org/2012/v17/af/3911/fulltext.asp?bframe=figures.htm&doi=yes



Das et al state regarding the above pathway model:

Schematic diagram for mda-9/syntenin mediated NFκB activation. Upon interaction with ECM (fibronectin), MDA-9/syntenin activates the p38/MAPK by augmenting FAK phosphorylation. This results in degradation of IκBα and movement of p65 from the cytoplasm where interaction with p50 results in binding to target genes (MT1-MMP) resulting in enhanced production of MT1-MMP, which interacts with TIMP-2 activating pro-MMP-2 to produce active MMP-2. This product then enhances cell motility, invasion, and cancer cell growth. mda-9/Syntenin activates the NF-kB pathway.

The original Figure appears to be from Boukerche et al as shown with some mods below:



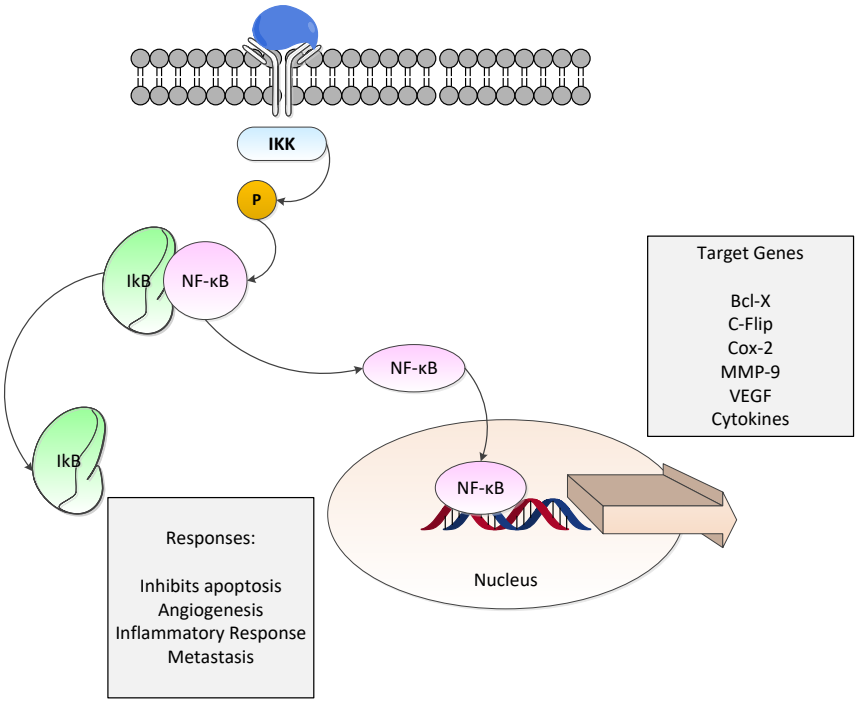
Note the differences. First the original shows multiple integrins and multiple FAK binding and in turn a binding of MDA-9 initiating the p38 pathway. Also note the explicit presence of NF-κB and its result of genes forcing mobility, invasion and metastasis. The authors state:

Hypothetical model of signal transduction pathways coordinately regulated by MDA-9/syntenin through its interaction with c-Src. MDA-9/ syntenin interaction with c-Src results in clustering of c-Src/FAK signaling complexes at high concentrations on the plasma membrane. The activated c-Src/FAK complexes activate the p38 MAPK/NF-κB pathways that regulate expression of genes involved in migration and invasion and Thus, play a crucial role in MDA-9/syntenin-mediated tumor progression.

The initiation of NF-κB is a significant factor since this transcription factor is what appears to be the instigator of the metastatic processes.

From Pecorino, p 220, we have again presented the details (as modified)[16]:

[16] Pecorino, Molecular Biology of Cancer, Oxford (New York) 2nd Ed, 2005.



The above graphic clearly demonstrates the movement of the transcription factor into the nucleus, from a bound state with IkB to an unbound and active state. The target genes indicated includes an MMP gene which again goes into the ECM.

As Sarkar et al state:

*Melanoma differentiation associated gene-9 (*mda-9*), also known as syntenin, is a PDZ domain–containing adapter protein that is involved in organization of protein complexes in the plasma membranes, regulation of B-cell development, intracellular trafficking and cell-surface targeting, synaptic transmission, and axonal outgrowth. Recent studies now define a seminal role for* mda-9*/sytenin in cancer metastasis*.

Thus, Sarkar who is part of Fisher's Lab at Virginia, have had a focus on Mda-9. They continue:

Adapter proteins play an essential role in modulating signal transduction from the extracellular environment to the intracellular milieu by virtue of their association with key regulatory molecules …

mda-9 was originally cloned as a gene differentially expressed in human melanoma cells reprogrammed to terminally differentiate by combination treatment with IFN-h and the protein kinase C activator mezerein … Analysis of the subcellular distribution of mda-9/syntenin revealed its localization at the areas of cell-cell contact in cells of epithelial origin in colocalization with F-actin, syndecan-1, E-cadherin, h-catenin, and a-catenin.

In fibroblasts, mda-9/ syntenin localizes to focal adhesions and in stress fibers. Overexpression of mda-9/syntenin in different cells induces the formation of plasma membrane structures,

including ruffles, lamellipodia, fine extensions, and neurite-like structures, showing its role in regulating the structure and function of the plasma membrane…

They continue:

The major characteristic of malignant tumor cells is their ability to invade foreign tissues and form metastatic foci at distant locations in the body. Such a process requires tumor cell attachment to various matrix proteins, degradation of the extracellular matrix (ECM) mainly by matrix metalloproteinases (MMP), followed by migration into the surrounding stroma by tumor cells…A model of progression of melanoma suggests that it begins by conversion of a normal melanocyte into a benign nevi, subsequent transformation into a radial and then a vertical growth phase primary melanoma, and finally evolution into a metastatic melanoma.

Finally, Sarkar et al outline the overall set of functions which MDA-9 is involved in. Specifically, they state:

1. ***Interleukin-5 signaling.*** *mda-9/syntenin interacts with interleukin- 5 (IL-5) receptor α and the transcription factor Sox4, Thus, mediating IL-5–induced Sox4 activation …*

2. ***Cell-surface trafficking.*** *Although mda-9/syntenin is located predominantly in the plasma membrane, it is also identified in the early secretory pathway such as the endoplasmic reticulum, intermediate compartment, and cis-Golgi, Thus, facilitating cellsurface trafficking of secreted molecules such as proTGF-α, an epidermal growth factor receptor ligand…*

3. ***mda-9/syntenin and ephrin signaling.*** *Ephrins and their cellsurface tyrosine kinase receptors are implicated in controlling axon guidance and fasciculation …*

4. ***Mediation of cohesiveness of epidermal stem cells.*** *In the basal layer of interfollicular epidermis the stem cells are clustered, a feature known as cohesiveness. These cells express high levels of Notch ligand D1, which is important for maintaining cohesiveness …*

5. ***Regulation of glutamate signaling.*** *The excitatory neurotransmitter glutamate interacts with its cognate receptors and regulates postsynaptic excitatory currents. Glutamate receptors interact with mda-9/syntenin, …*

6. ***Regulation of axon outgrowth.*** *Unc51.1 is a serine/threonine kinase that is important for neurite extension/parallel fiber formation in cerebellar granule neurons. mda-9/syntenin interacts with Unc51.1 and Rab5, a member of the Ras-like small GTPases that is a marker of early endosomes and is essential for endocytic membrane fusion and trafficking. …*

Boukerche et al (2005) stated:

Studies using an enhanced green fluorescent protein mda-9/ syntenin fusion protein showed that endogenous mda-9*/syntenin colocalized with the E-cadherin complex and syndecan-1 at adherens junctions as well as with focal adhesions and stress fibers at cell-substratum contact in*

fibroblastic and epithelial cells. These findings suggest that Mda-9*/syntenin might promote cytoskeletal organizational changes and intracellular signaling.*

The organization of these dissimilar focal contacts is complex but was shown not only to contain the appropriate integrin but also cytoskeletal proteins (vinculin, talin, and a*-actinin) as well as several cytoplasmic protein tyrosine kinases, including members of the src family and focal adhesion kinase (FAK). Despite extensive research documenting an ability of* mda-9*/syntenin to form multivalent interactions, little is known about the role of* Mda-9*/syntenin in cancer development.*

Boukerche et al (2008) state:

Prior studies confirm that Mda-9*/syntenin stimulates motility through pathways involving FAK, p38MAPK, and NF-κB, leading to secretion of MMP-2 (4, 9). However, despite these intriguing observations, it is not fully understood how* Mda-9*/syntenin orchestrates these signaling molecules to enhance cancer cell motility and metastasis. A complex network of protein-protein interactions characterizes the structural organization of focal adhesions, involving known signaling molecules that play functional roles in various cellular activities and other less well-defined pathways.*

We presently show that Mda-9*/syntenin interacts with c-Src through its PDZ domain and activates the c-Src/FAK signaling pathway to maximize tumor cell motility and anchorage-independent growth of melanoma cells.* Mda-9*/Syntenin levels directly correlate with increased c-Src activity in a human melanoma model that closely mimics the early events of metastasis in humans.*

In 2010 Boukerche et al report:

MDA-9/syntenin affects cancer cell motility and invasion through distinct biochemical and signaling pathways, including focal adhesion kinase and p38 mitogen-activated protein kinase (MAPK), resulting in activation of the nuclear factor (NF)-kappaB pathway.

MDA-9/syntenin also promotes melanoma metastasis by activating c-Src, but how c-Src regulates NF-kappaB activation is unclear. Using a human melanoma model, we document that MDA-9/syntenin-c-Src interactions are positive regulators of NF-kappaB activation. Inhibition of c-Src by PP2 treatment, by blocking c-Src or mda-9/syntenin expression with small interfering RNA, or in c-Src (-/-) knockout cell lines, reduces NF-kappaB activation following overexpression of mda-9/syntenin or c-Src.

Deletion or point mutations of the PDZ binding motif preventing MDA-9/syntenin association with c-Src reveals that both PDZ domains, with PDZ2 being the dominant module, are required for activating downstream signaling pathways, including p38 MAPK and NF-kappaB. We also document that MDA-9/syntenin-c-Src complexes functionally cooperate with NF-kappaB to promote anchorage-independent growth, motility and invasion of melanoma cells.

These findings underscore PDZ domains of MDA-9/syntenin as promising potential therapeutic targets for intervening in a decisive component of cancer progression, namely, metastatic tumor spread.

4.6 OBSERVATIONS

This set of papers from the Fisher Lab present several interesting connections between the ECM and the intra-cellular signaling paths. We have had prior arguments that one can develop models for metastasis by examining the cell as a target entity and then by modeling the environment, both the ECM and surrounding cells as influences on the target cell. In this work we can expand it to include ECM factors in some detail.

The suggested control of other pathway elements, beyond just the B-RAF control that we now have may be proven productive. Notwithstanding it does establish a research path that is based upon established cell dynamics.

5 TUMOR MICROENVIRONMENT (TME)

Tumors are not self-sufficient entities. They are not aberrant cells awash in a sea of normalcy. The create and in a sense are created by the microenvironment in which the grow and expand. We have examined some of the specific protein elements of the ECM and now we will examine the cellular components.

5.1 OVERVIEW

We begin with a brief discussion of the TME and then will proceed with some details. As Roma-Rodrigues et al have noted:

The development of effective anti-cancer therapies has been challenged by the overall complexity of tumors. The tumor heterogeneity is exacerbated during the progression of the cancer along with the maturation of the cellular and noncellular components of the tumor niche—the tumor microenvironment (TME).

The TME consists of extracellular matrix (ECM), stromal cells (such as fibroblasts, mesenchymal stromal cells, pericytes, occasionally adipocytes, blood and lymphatic vascular networks) and immune cells (including T and B lymphocytes, natural killer cells and Tumor-associated macrophages).

The TME has progressively been shown to dictate aberrant tissue function and to play a critical role in the subsequent evolution of malignancies. Epithelial tumors display common features that allow for the setting of hallmarks that define cancer progression. Tumor initiation is based on a complex series of biological events occurring on a normal cell that will result in hyperplasia, uncontrolled growth and resistance to cell death. As tumor cells continue proliferation, the tumor increases in size with an associated remodeling of the TME. This is induced by hypoxia, oxidative stress and acidosis, due to an alteration of tumor cells metabolism, resulting in dysplasia, which is the appearance of a heterogeneous population of tumoral cells with different genetic and phenotypic traits.

These events are orchestrated by autocrine and paracrine communications with stromal cell and immune system adjacent to the tumor, coupled to an increased interstitial fluid pressure.

Once again, autocrine and paracrine communications between TME cells induce TME maturation and tumor progression, resulting in increased stiffness of the extracellular matrix, formation of blood and lymph vessels, possible appearance of necrotic regions and metastasis.

5.2 TME AND IMMUNE SYSTEM

As Whiteside had noted:

The tumor microenvironment is created by the tumor and dominated by tumor-induced interactions. Although various immune effector cells are recruited to the tumor site, their anti-tumor functions are downregulated, largely in response to tumor-derived signals. Infiltrates of

inflammatory cells present in human tumors are chronic in nature and are enriched in regulatory T cells (Treg) as well as myeloid suppressor cells (MSC).

Immune cells in the tumor microenvironment not only fail to exercise antitumor effector functions, but they are co-opted to promote tumor growth. Sustained activation of the NF-jB pathway in the tumor milieu represents one mechanism that appears to favor tumor survival and drive abortive activation of immune cells. The result is tumor escape from the host immune system.

Tumor escape is accomplished through the activation of one or several molecular mechanisms that lead to inhibition of immune cell functions or to apoptosis of anti-tumor effector cells. The ability to block tumor escape depends on a better understanding of cellular and molecular pathways operating in the tumor microenvironment. Novel therapeutic strategies that emerge are designed to change the protumor microenvironment to one favoring acute responses and potent anti-tumor activity

The author continues:

A tissue microenvironment of developing tumor is comprised of proliferating tumor cells, the tumor stroma, blood vessels, infiltrating inflammatory cells and a variety of associated tissue cells.

It is a unique environment that emerges in the course of tumor progression as a result of its interactions with the host. It is created by and at all times shaped and dominated by the tumor, which orchestrates molecular and cellular events taking place in surrounding tissues. Immune cells present in the tumor include those mediating adaptive immunity, T lymphocytes, dendritic cells (DC) and occasional B cells, as well as effectors of innate immunity, macrophages, polymorphonuclear leukocytes and rare natural killer (NK) cells.

NK cells, which mediate innate immunity and are rich in perforin- or granzyme-containing granules, are conspicuously absent from most tumor infiltrates or even pre-cancerous lesions. Although NK cells represent 'the first line' of defense against pathogens and mediate potent antitumor cytotoxicity in vitro, in tumor milieu, they are infrequent, despite the fact that tumor cells frequently downregulate expression of HLA antigens and are enriched in MICA and MICB molecules. ...

TIL clones with the specificity to a broad variety of the tumor-associated antigens can be outgrown from human tumors, confirming that immune responses directed not only at 'unique' antigens expressed by the tumor, but also at a range of differentiation or tissue-specific antigens, are generated by the host.

Although accumulations of these effector T cells in the tumor might be considered as evidence of immune surveillance by the host, they are largely ineffective in arresting tumor growth. Among CD4 + T cells present in the tumor, a subset of CD4+ CD25 high Foxp3 + cells is expanded (5–15% of CD3 +CD4+þ T cells in TIL) relative to their significantly lower frequency in the peripheral circulation of patients with cancer. These cells are regulatory T cells (Treg) capable

of suppressing proliferation of other T cells in the microenvironment through contact-dependent mechanisms or IL-10 and TGF-b secretion. They come in different flavors (for example, nTreg, Tr1) and are a characteristic feature of the microenvironment in human tumors.

Macrophages present in tumors are known as tumor associated macrophages or TAMs. They are re-programmed to inhibit lymphocyte functions through release of inhibitory cytokines such as IL-10, prostaglandins or reactive oxygen species (ROS).

Myeloid suppressor cells (MSC) accumulating in human tumors are CD34+ CD33 + CD13+ CD15- bone marrow-derived immature dendritic cells, an equivalent to CD11b þ/ Gr1 þ cells in mice. They promote tumor growth and suppress immune cell functions through copious production of an enzyme involved in L-arginine metabolism, arginase 1, which synergizes with iNOS to increase superoxide and NO production, blunting lymphocyte responses (Ochoa et al., 2007) and by induction of iNOS in surrounding cells. …

Polymorphonuclear leukocytes are infrequently seen in infiltrates of human tumors, with the exception of nests of eosinophils that may be present in association with tumor cells in various squamous cell tumors, for example. In contrast, granulocytes tend to be a major cellular component of many murine tumor models. This disparity may be because of a different nature of infiltrates, which in man are chronic rather than acute. Acute cellular responses may be long gone by the time human tumors are diagnosed, biopsied and examined.

Inflammatory cells present in the tumor microenvironment either contribute to tumor progression or actively interfere with its development. It is clear today that the former takes precedence, largely because the tumor generally proceeds to establish mechanisms responsible for its 'immune evasion' or escape from the immune intervention.

The tumor not only manages to escape from the host immune system, but it effectively contrives to benefit from infiltrating cells by modifying their functions to create the microenvironment favorable to tumor progression. To this end, immune cells infiltrating the tumor together with fibroblasts and extracellular matrix forming a scaffold supporting its expansion, contribute to establish an inflammatory milieu that nourishes the tumor and promotes its growth. Tumor escape from the host is facilitated by the ability of human tumors to actively subvert antitumor immunity by downregulating or completely suppressing local and systemic innate as well as adaptive antitumor immunity by a variety of mechanisms as discussed below.

Recently Arneth noted:

The TME refers to the cellular environment in which tumors or cancer stem cells exist. Cancer stem cells are cells in a tumor with the abilities to self-renew and drive tumorigenesis.

Previous studies have isolated unique cancer stem cells in samples from patients with breast, hematopoietic, colon, lung, and brain cancers.

These cells help improve the understanding of the TME, but pose significant challenges in the diagnosis and management of cancer.

The TME encompasses the surrounding immune cells, blood vessels, extracellular matrix (ECM), fibroblasts, lymphocytes, bone marrow-derived inflammatory cells, and signaling molecules.

Interactions between malignant and nonmalignant cells create a TME that affects cancer development and progression. The nonmalignant cells in the TME often play a protumorigenic function at all phases of carcinogenesis by stimulating uncontrolled cell proliferation. In contrast, malignant cells invade healthy tissues and spread to other body parts through the lymphatic or circulatory system.

The TME comprises different cellular components.

The first is endothelial cells, which play a key role in tumor development and tumor cell protection from the immune system.

Tumor angiogenic vessels usually branch outwards from preexisting vessels or are derived from endothelial progenitor cells. In this way, these cells offer nutritional support for tumor growth and development.

The second major component is immune cells, such as granulocytes, lymphocytes, and macrophages.

These cells are involved in various immune responses and activities, such as inflammatory reactions orchestrated by the tumor to promote survival. The most prominent immune cell type in the TME is the macrophage.

Macrophages have diverse functions that are linked to cancer development and progression; they promote the escape of tumor cells into the circulatory system and can suppress antitumor immune mechanisms and responses.

Evidence from previous studies has revealed that macrophages can help circulating cancer cells extravasate at distant sites, such as the lungs, which can lead to the persistent growth of metastatic colonies. An increasing number of studies have revealed that tumor-associated macrophages (TAMs) can augment, mediate, or antagonize the antitumor activity of irradiation, cytotoxic agents, and checkpoint inhibitors. The final cell type in the TME is the fibroblast.

Fibroblasts allow cancer cells to migrate from the primary tumor location into the bloodstream for systemic metastasis. Furthermore, fibroblasts provide a reliable passage for endothelial cells undergoing angiogenesis in the tumor.

The role of the ECM in cancer development and progression has been examined in previous studies. The ECM consists of a network of macromolecules, including glycoproteins, collagens, and enzymes, that support biomechanical activities and functions in the body. Importantly, the ECM is composed of active tissue components that influence cell adhesion, proliferation, and communication.

The cellular growth factors found in the matrix near other cell membranes, such as integrins, are implicated in the ability of cells to communicate with the TME. The ECM further influences the migration of cancer cells by altering its physical properties, composition, and topography. The adhesion gradient and the ECM concentration determine the speed at which cancer cells migrate from one region to another.

5.3 Detailed Elements of TME

From Lau et al the stromal cells in the TME consist of the following:

5.3.1 Cancer-Associated Fibroblasts.

Cancer-associated fibroblasts (CAFs) are the major components of the tumor stroma. Recent studies have revealed that CAFs are a heterogeneous population, most of which acquire the activated phenotype with increased contractile force, proliferative activity, and enhanced secretion of ECM, proteases, and growth factors. CAFs emerge from multiple origins that widely vary among different cancer types. Several studies have shown that cancer cells could actually secrete signaling molecules, such as basic fibroblast growth factor (bFGF), transforming growth factor beta (TGF-β), platelet-derived growth factor (PDGF), and interleukin IL-6 to "educate" resting fibroblasts to become CAFs, and in turn, CAFs promote tumor growth and sustain the stemness property of CSCs in a paracrine manner.

Through the secretion of hepatocyte growth factor (HGF), CAFs from colon cancer were demonstrated to support CSC properties through the induction of Wnt/β-catenin signaling. More interestingly, the paracrine activation of Wnt/β-catenin signaling by CAFs could restore the stem-like features of non-CSCs, thereby expanding the pool of these cells.

Using conditioned media from CAFs, we showed that CAFs from liver cancer promote cancer stemness through the noncanonical induction of the Notch signaling effector HEY-1 mediated by HGF. A recent study also demonstrated that CAFs in lung cancer induce the expression of the NANOG transcription network through paracrine insulin-like growth factor II (IGF-II)/IGF- 1R signaling. EMT is the process where cancer cells acquire a mesenchymal trait and become more invasive and metastatic.

Cancer cells that have undergone EMT typically acquire an increased stemness property because some of the EMT-mediating transcription factors, such as Snail and ZEB1, are essential for self-renewal. Several studies have also shown that the activation of EMT could induce the generation of the CSC population.

In prostate cancer, CAFs can elicit EMT and increase the stemness properties of cancer cells through the secretion of MMPs. Furthermore, CAFs from breast cancer have been reported to promote the EMT of cancer cells via the secretion of stromal-derived factor 1 (SDF-1) and TGF-β1, providing additional support, suggesting that CAFs play a crucial role in promoting cancer stemness.

5.3.2 Adipocytes.

Obesity is a well-recognized risk factor of several common human malignancies, including breast cancer, colon cancer, and liver cancer.

In addition to its epidemic significance, emerging studies have uncovered the functional role of adipose tissues in carcinogenesis and cancer progression, particularly in cancers with adipose tissue constituting a major part of the tumor microenvironment.

Adipose tissue primarily comprises adipocytes and a variety of cells that make up the stromal vascular fraction. In addition to its lipid storage function, adipocytes can actively secrete multiple adipokines and cytokines, such as leptin, adiponectin, IL-6, MCP-1, and TNF-α, during excessive adiposity. In addition to its role in lipid homeostasis, many of these adipokines and cytokines are proinflammatory, which attract the infiltration of inflammatory cells, particularly macrophages, causing chronic inflammation to promote cancer growth and metastasis.

Furthermore, some of these adipocyte-secreted adipokines/cytokines were directly involved in regulating CSCs. In breast cancer, the expression of leptin receptor is highly upregulated in tumor tissue, particularly in the CSC subpopulation, as driven by the selfrenewal associated transcription factors OCT-4 and SOX-2. The secretion of leptin by adipocytes activates the STAT3 signaling in CSCs and induces the expression of OCT-4 and SOX-2, in turn stimulating the expression of leptin receptor, which maintains a self-reinforcing signaling cascade to expand the CSC population and promote tumor growth.

Another study showed that the coculture of adipocytes and breast cancer cells stimulates the production of various cytokines that promote cancer stemness through the Src/SOX-2/miR-302b signaling pathway. In prostate cancer, where obesity is associated with a more aggressive phenotype, adipocytes produce cathepsin B (CTSB) upon coculture with prostate cancer cells to support the selfrenewal of CSCs. Adipocytes from colorectal cancer are also demonstrated to enhance cancer stemness, and their oncogenic function can be impaired by grape seed extract, a well proven agent with anticolorectal cancer activity, through inducing the "browning" of adipocytes.

5.3.3 Perivascular Cells.

Angiogenesis is essential for tumor growth and metastasis. With the excessive production of proangiogenic factors by cancer cells, tumors typically develop disorganized and rich blood vessel networks to meet the high demand on oxygen and nutrients required for tumor outgrowth. CSCs promote tumor angiogenesis.

For example, in brain, skin, pancreatic, and liver cancer, the CD133+ CSC populations produce higher levels of proangiogenic factors, such as vascular endothelial growth factor (VEGF) and SDF-1, recruit more endothelial cells, and stimulate more tube formation compared with their differentiated CD133− counterparts. Intriguingly, glioblastoma stem cells, which reside in the perivascular niche, undergo differentiation to generate vascular pericytes and endothelial cells to expand tumor vascularization.

Indeed, a mean of approximately 60% of endothelial cells in glioblastoma are derived from neoplastic cells. In turn, CSCs reside in close proximity to the perivascular niche, which provides functional support. Strong evidence suggests that vascular endothelial cells play a key role in maintaining CSCs. In the context of glioblastoma, endothelial cells provide Notch ligands to neighboring CSCs, activating Notch signaling and promoting CSCs self-renewal. In another study, perivascular endothelial cells were demonstrated to activate Notch signaling in glioma stem cells through another soluble factor, nitric oxide.

A similar observation was also made in colon cancer, suggesting that endothelial cells secrete the Notch ligand Jagged-1 to promote colon CSC phenotype. A recent study on head and neck cancer also highlighted a role for endothelial cells in regulating CSCs, in which endothelial cells were shown to secrete epidermal growth factor (EGF) to induce EMT and promote cancer stemness. Together, these findings reveal an intriguing reciprocal interaction between CSCs and perivascular cells.

5.3.4 CSCs and Immune Evasion.

Tumor immune escape is a fundamental step for tumor development and the major reason for the failure in cancer immunotherapy. Cancer cells evade the infiltration and the cytotoxic function of natural killer (NK) T cells and CD8+ cytotoxic T cells through various strategies, including the active attraction of immunesuppressive cells, production of immune-suppressive factors, and the activation of "immune checkpoints" that induce anergy or apoptosis in T lymphocytes to downmodulate immune functions.

Several studies have revealed that the activation of prosurvival pathways, such as PI3K/AKT, in CSCs not only facilitates escape from conventional chemotherapies but also confers immune evasion. The expression of MHC-I and MHC-II proteins, required for recognition by T lymphocytes to elicit immune responses, is also downregulated in CSCs. In head and neck cancer, the programmed death-ligand 1 (PD-L1), which binds to the programmed death 1 (PD-1) receptor on T cells to suppress its function, is selectively expressed on CD44+ CSCs [42]. Furthermore, it has been well documented that CSCs actively recruit immune-suppressive cells into the tumor microenvironment.

In addition to functions in modulating immune cells, these tumor-associated immune-suppressive cells, which mainly include tumor-associated macrophages myeloid-derived suppressor cells (MDSCs), T-regulatory (Treg) cells, and NK cells, have been widely demonstrated to support CSCs through multiple pathways.

5.3.5 Tumor-Associated Macrophages.

The TAMs have been found to play a significant role in facilitating cancer cell proliferation. M1 and M2 macrophages can counter one another as well as transform from one to the other. The author notes:

Macrophages are classified into M1- and M2-polarized subtypes. The M1-subtype secretes inflammatory cytokines and reactive oxygen intermediates and presents antigen to tumor suppressive T cells.

However, the M2-subtypes, which are tumor promoting, induce T cell anergy, produce extracellular matrix components, repair damaged tissues, and induce angiogenesis. Although the origins of macrophages in many cancers remain uncertain, most of the macrophages recruited to the tumor microenvironment, known as the TAMs, become the tumor supportive M2 subtype. In glioblastoma, glioma CSCs activate the STAT3 pathway to produce cytokines, which recruit and polarize macrophages to become M2-like.

After recruitment, TAMs, in turn, serve as a CSC niche to support CSC growth. For example, in breast cancer, the physical interaction between TAMs and CSCs activates the EphA4 receptor on CSCs and the downstream Src and NF- κB pathways, which promote self-renewal.

5.3.6 Myeloid-Derived Suppressor Cells.

MDSCs are a heterogeneous population of myeloid-originated progenitor cells. ...As the name indicates, the main feature of MDSCs is their function on immunosuppression. MDSCs suppress immune function primarily through multiple mechanisms, including the production of arginase, inducible nitric oxide synthase (iNOS), reactive oxygen species (ROS), cyclooxygenase-2 (COX-2), and TGF- β, which together inhibit the proliferation and function of T cells.

Recent studies have demonstrated that MDSCs are actively recruited into tumors and these tumor-associated MDSCs play an important role in tumor progression. The recruitment of MDSCs into tumor sites is primarily mediated by various cancer cells that produce chemokines, including CCL2, CCL15, CXCL5, and CXCL12. MDSCs are implicated in multiple stages of tumor progression, particularly the regulation of CSCs. In ovarian cancer, coculture with MDSCs stimulates the expression of miR-101 in cancer cells, which regulates CtBP2 to control the expression of stemness genes, such as NANOG, OCT-4, and SOX-2.

In syngeneic mammary tumor models, CSCs displayed the elevated production of granulocyte colony-stimulating factor (G-CSF), which stimulates the recruitment of MDSCs into the tumor microenvironment. MDSCs reciprocally enhance CSC properties through the activation of Notch signaling. Furthermore, tumor-infiltrated MDSCs, which showed the activation of STAT3 signaling, can enhance the stemness of pancreatic cancer cells through the induction of EMT, with a concomitant increase in the expression of stemness genes, including Snail, Slug, ZEB1, NANOG, and OCT-4.

5.3.7 T-Regulatory Cells.

The fine cross talk between CSCs and immunosuppressive cells also involves Treg cells. Treg cells are defined by the CD4+CD25+FOXP3+ T cell subpopulation, with FOXP3 as an important transcriptional regulator of Treg cell development and function. Treg cell-mediated immunosuppression primarily occurs through the production of various cytokines, such as IL-10, IL-35, and TGF-β, direct cell-cell contact via gap junctions, or metabolic disruption in which

CD39 and CD73, expressed on Treg cells, facilitate the conversion of ATP to adenosine, which suppresses cytotoxic T cell and/or NK cell activity.

In tumors, Treg cells are accumulated by various mechanisms, primarily involving chemokine attractions. For example, the chemokines CCL22 and CCL28 are produced by tumor cells to attract CCR4- and CCR10-expressing Treg cells, respectively, leading to the accumulation of Treg cells in various human cancers. Indeed, the number of Treg cells inside the tumor microenvironment is associated with clinical outcome. The higher number of Treg cells within the tumor is correlated with poor prognosis in a wide array of cancers, including gastric, esophageal, pancreatic, liver, and breast cancers. In addition to its immune-suppressive role, the functional importance of tumor-infiltrating Treg cells in regulating CSCs is starting to emerge.

A recent report demonstrated that, under hypoxia, FOXP3+ Treg cells are induced to express IL-17, which drives the expansion of CSCs through the activation of Akt and MAPK signaling pathways in colorectal cancer, evidenced by the increase in the expression of colorectal CSC markers, including CD133, CD44s, and EpCAM. Furthermore, Treg cells produce and secrete prostaglandin (PGE2) for immunosuppression, and PGE2 has been implicated in the regulation of CSC properties in colorectal cancer through NF-κB.

5.3.8 Natural Killer Cells.

NK cells are often the first to attack aberrant intruders including cancer cells. As part of the innate immune system they can be effect first remediation players. However NK cells can be co-opted as are other immune elements. The author notes:

The ability of natural killer (NK) cells to kill or spare depends on their expression of activating (mostly stress-induced proteins) and inhibitory (in particular MHC class I molecules) ligands on the surface of target cells.

Approximately 95% of peripheral blood NK cells are CD56dim CD16+ which exerts strong cytotoxic activity. The remaining 5% of peripheral blood NK cells are CD56bright CD16− and show cytotoxicity through strong cytokine production. CD133+ glioblastoma stem cells that are able to express high levels of the activating DNAM-1 ligands PVR and Nectin-2 and low levels of MHC class I molecules have been reported to be poorly recognized and lysed by NK cells. Their cytotoxic activity was revamped following IL-2 or IL-15 activation.

Breast cancer CSCs have also been reported to fail to express detectable levels of NK ligands, which is consistent with metastatic spread. In melanoma and GBM, CSCs are highly resistant to NK cells and become susceptible to NK cytotoxicity only following stimulation with IL-2. However, the preferential resistance of CSC to NK cells is not the rule, as colon CSCs express lower MHC class I and higher levels of NK-activating ligands, including NKp30L and Nkp44L as compared to differentiated cells, which are responsible for the CSC susceptibility to NK cell killing.

Another mechanism by which cancer cells may evade from the cytotoxic effect of NK cells is the induction of apoptosis in microenvironmental immune cells through the interaction of

CD95 (Apo1/Fas) with its ligand (CD95L). Interestingly, CD95R/L regulates CSC plasticity and its blockade reduces CSC in different tumor cell models, while activation of CD95R/L increases CSC number and is responsible for CSC reduced sensitivity to CD95-mediated apoptosis.

Collectively, CSCs are more refractory to the cytotoxic effect of NK cells in a variety of cancer types.

5.3.9 Other Stromal Cells.

There is increasing evidence that mast cells (MCs) and their mediators are involved in the remodeling of the tumor microenvironment. Recent evidence has showed that MC regulates stemness of thyroid cancer through IL-8-Akt-Slug pathway. In prostate cancer, MC increased stem/progenitor cell population via altering LncRNA-HOTAIR/PRC2-androgen receptor- (AR-) MMP9 signals. In addition, neutrophils were found to play a crucial role in regulation of CSC populations. …

Hypoxia Hypoxic microenvironments in tumors result from the rapid growth of cancer cells, which exceeds the limit of blood supply. In response to the hypoxia, the hypoxia-related gene expression is driven through the activated hypoxiainducible factor (HIF) and transcription factors HIF-1α and HIF-2α that bind to the hypoxia-regulated element (HRE) gene promoters. The capacity of HIFs to promote cancer cell stemness has been well documented. Studies have shown that HIFs can increase the expression of stem cell markers in breast cancer.

Bae et al. demonstrated that hypoxia can elevate the expression of the stem cell marker SOX2 in prostate cancer cell lines. In addition, the overexpression of HIF-1α has been associated with stem cell marker CD44 in bladder cancer. In addition to HIFs, the hypoxia-mediated overexpression of extracellular carbonic anhydrases, CAIV and CAXII, facilitates cancer cell survival and the maintenance of CSC function. Given that CSC is related to metastasis and cancer cell invasion, the contribution of hypoxia to the enhanced CSC migration has been reported in several studies.

The upregulation of EMT-related gene expression under hypoxic stress can enhance the invasiveness and the stem-like properties of cancer. Maeda et al. showed that HIF-1α is correlated with the EMT and cell migration in CD133+ pancreatic CSCs. In addition to cancer cell invasion, hypoxia contributes to drug resistance by maintaining CSCs in a quiescent state to confer resistance to chemotherapeutics that commonly target actively dividing cancer cells.

Studies have reported that hypoxia promotes SOX-2-mediated drug resistance in ovarian CSCs via Notch signaling.

The downregulation of HIF-1α using a lentivirus-mediated approach can increase the chemosensitivity in triple negative breast cancer. These data demonstrated that hypoxia plays an important role in the CSC niche and is substantially involved in the regulation of cancer cell stemness.

6 ADIPOCYTES

Adipocytes are the cells which store fat and in turn energy for the body. Excess adipocytes and excess fat leads to obesity and in addition this condition can initiate and enhance a variety of malignancies. This is one of the major reasons we see a proliferation of obesity related morbidities, especially malignancies, as the population has increasing rates of obesity.

6.1 OVERVIEW

Over the last few decades a considerable amount of research has helped to clarify the effect that excess adipocytes have on increasing malignancies. To best understand some of these issues it is worth examining the types of adipocytes, their functions and their actions on the immune system as well. We start with Gupta who presents an excellent overview of adipocytes:

In vertebrates, many cell types can accumulate lipid; However, the evolution of specialized fat-storing cells ('white adipocytes') has provided a safe and specific compartment for this purpose. White adipocytes are characterized by the presence of a single large lipid droplet and are therefore also known as 'unilocular' adipocytes. Their classical function, first and foremost, is to serve as an energy bank.

During times of energy excess, free fatty acids (FFAs) enter adipocytes following the hydrolysis of triglycerides from triglyceride-rich lipoproteins and chylomicrons. FFAs are then re-esterified into triglycerides through the sequential actions of multiple enzymes, including glycerol-3-phosphate acyltransferase (GPAT), 1-acylglycerol-3-phosphate acyltransferase (AGPAT), phosphatidic acid phosphatase (PAP), and diacylglycerol acyltransferase (DGAT). Adipocytes can also synthesize lipid from carbohydrates through de novo lipogenesis. When energy levels are low, adipocytes contain the enzymatic machinery — comprising adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), and monoglyceride lipase (MGL) — required to hydrolyze triglycerides and release FFAs back into circulation.

Lipid trafficking in adipocytes has been extensively studied for the past decade; However, there remains much to learn. The principle enzymes in adipocyte triglyceride metabolism have been identified, although the precise function and relative importance of these enzymes and their distinct isoforms in vivo remain unclear. A better understanding of how these mechanisms are utilized in human adipose tissue, and how they are dysregulated in metabolic disease, will also be essential.

More recently, it has become clearer that proteins associated with the lipid droplet, such as the perilipin family of proteins, play an important role in regulating lipolysis and lipid metabolism in adipocytes. Understanding the precise functions of these various proteins and how they communicate with other pathways within the cell is of great interest. Furthermore, lipid intermediates themselves can serve as signaling molecules in adipocytes, through binding to nuclear hormone receptors or interacting with second-messenger systems. Identifying and characterizing these various lipid signals is an ongoing challenge in the field.

6.1.1 Endocrine role.

For nearly a century, adipocytes were appreciated solely for their energy storage capacity. This view began to change with the discovery that adipose tissue in obesity produced tumor necrosis factor a (TNF-a), a pro-inflammatory cytokine that drives insulin resistance; this provided the first link between adipose-secretory products and insulin resistance in obesity.

***The discovery of the hormone leptin was a pivotal point in the field of energy metabolism. Leptin is produced and secreted by adipocytes and functions centrally to regulate satiety**. Leptin also acts on peripheral tissues to control nutrient homeostasis and inflammation. Importantly, this discovery provided evidence of a now widely appreciated endocrine role for adipocytes. Contemporaneously, t**wo other 'adipokines' (adipocytederived cytokines) — adiponectin and adipsin** — were identified.*

***Adiponectin is produced almost exclusively by adipocytes and exerts powerful and pleiotropic effects on glucose and lipid metabolism, and also provides cardioprotection.** Adipsin, also expressed exclusively in adipocytes, acts in an adipose–pancreas interorgan axis to regulate the insulinsecretory capacity of β-cells.*

The list of adipokines is growing and their widespread effects on energy balance, cardiovascular function, immune regulation, and nutrient homeostasis are becoming more and more evident. There is tremendous excitement and promise in understanding the wide range of physiological roles for these adipose secretory proteins because many adipokines exhibit therapeutic potential.

However, the complexity of their action also represents a challenge for the design of effective and specific treatments. Going forward, the specific mechanisms by which adiponectin, leptin, and other adipokines elicit their functions in different target tissues will need to be further elucidated. Moreover, the relative importance of many of these factors remains unclear. Targeted deletions of cognate receptors or target pathways in animal models will ultimately help provide insight into the precise roles of many of these adipokines.

*In addition to the energy-storing white adipocytes described above, all mammals have a **second major type of adipocyte that functions to convert chemical energy into heat.***

*These thermogenic **'brown adipocytes'** are characterized by their abundance of mitochondria (which give the cells their brown appearance) and multilocular fat droplet appearance.*

Distinct depots of brown adipose tissue (BAT) are most abundant in the interscapular region of small mammals and infants, and develop during embryonic development. Brown adipocytes likely evolved to help defend animals against the cold and were historically referred to as the 'hibernating organ' due to their function in maintaining body temperature in hibernating animals and newborns.

Brown-like adipocytes also appear within distinct white adipose depots as dispersed pockets of multilocular cells. These cells, now termed 'beige adipocytes', represent recruitable thermogenic cells that arise in response to cold challenge, exercise, and under pathological conditions such as cancer-associated cachexia. Beige fat cells play a meaningful role in the regulation of glucose homeostasis and energy balance. These cells, although thermogenic, appear to be molecularly and developmentally distinct from brown adipocytes that form during development (often termed 'classical BAT').

Adult humans contain appreciable amounts of active thermogenic adipose tissue; molecular analyses suggest that this tissue closely resembles the rodent beige fat, but also has some features of classical brown adipocytes. The thermogenic function of brown and beige adipocytes is mediated by the specific expression of uncoupling protein 1 (UCP1).

UCP1 is a transport protein that sits within the inner membrane of mitochondria and catalyzes a proton leak across the inner membrane, dissipating the electrochemical gradient that has been generated via the electron transport chain, thereby uncoupling oxidative metabolism from ATP synthesis. Heat production occurs as the biochemical reactions involved in mitochondrial fuel oxidation are subsequently accelerated. Tremendous effort is focused towards identifying 'druggable' regulators of UCP1 expression or activity.

In effect, we may know a great deal about adipocytes yet the complete understanding is still incomplete.

6.2 BASIC ADIPOCYTES

We briefly detail some of the key issues related to the two major types of adipocytes.

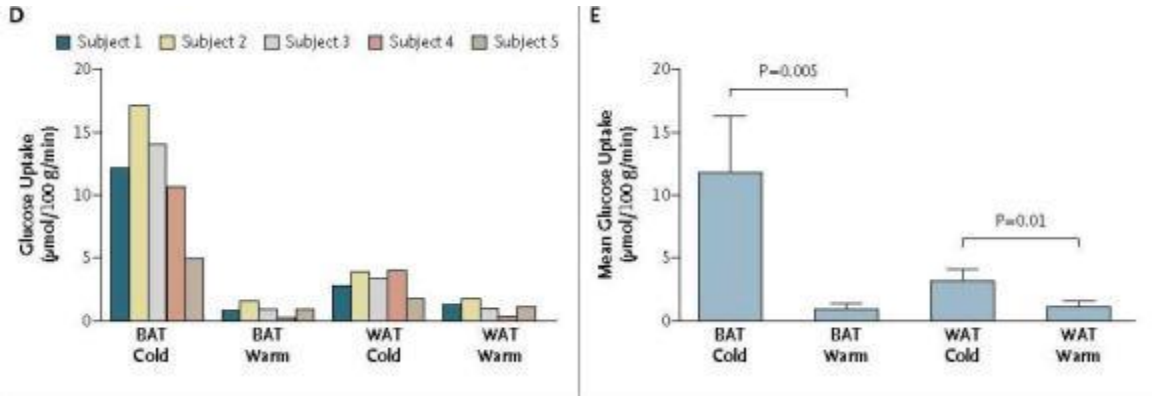
6.2.1 Brown Adipose Tissue

Brown Adipose Tissue, BAT, is the key cellular element in tissue thermogenesis, the production of thermal output. It has been thought to be only in infants and some other mammals and that it disappears in adults. As Hahn and Novak have stated, BAT was found to have a key role in non-shivering thermogenesis. The production of non-shivering heat is stimulated by norepinephrine.

However, as Virtanen et al state:

"Active brown adipose tissue helps maintain normal body temperature in newborn infants. It is believed that this tissue regresses with increasing age and is completely lost by the time a person reaches adulthood. However, the capacity to produce brown adipose tissue in adulthood has been shown in patients with catecholamine secreting tumors such as pheochromocytomas and paragangliomas, in whom distinct brown adipose tissue depots develop…"

From the Virtanen paper above the authors present a comparison between brown and white fat and the energy consumed or generated and it is depicted below:



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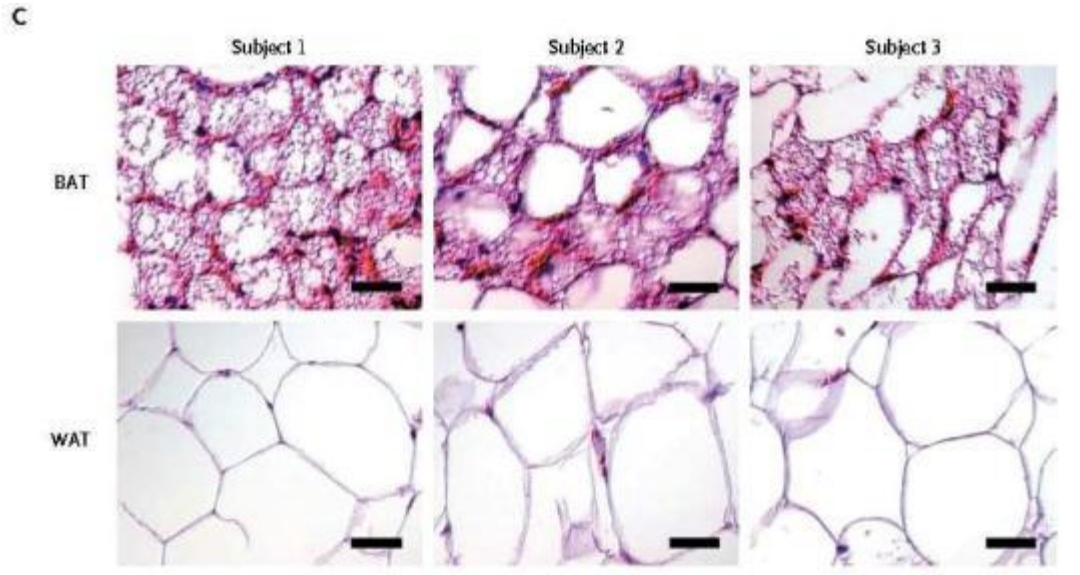
Clearly the brown fat burns energy at a much more rapid rate than white fat. That is most likely why it is so prevalent in infants and seems to regress, albeit not totally, in adults. The comparison between white and brown cells upon which this data was obtained is shown below:



In summary from the seminal work of Hahn and Novak we have the observation of BAT as follows:

In summary, BAT in neonatal mammals plays an important role in nonshivering heat production. Fatty acid oxidation is of prime importance. Nevertheless, glycolysis and Krebs cycle intermediates are necessary for normal BAT function. Partial uncoupling of mitochondria seems to be induced by released fatty acid. An interesting point is the very high activity of phosphoenolpyruvate carboxykinase. This suggests the possibility of a cycle between this enzyme and pyruvate kinase and might account for the dissipation of energy resulting from a decrease in the P/O ratio induced by increased cellular fatty acid content, while substrate level phosphorylation, which apparently is functional in BAT mitochondria, remains unaffected.

6.2.2 White Adipose Tissue

White Adipose Tissue takes in glucose and creates and store fats which may be used from time to time through lipolysis to run the TCA or Krebs cycle. We show below the cycle of glucose entering the cell, the TCA cycle processing it to produce the fats via fatty acid production and then when necessary the fats being broken down for energy in the TCA cycle.

In summary from the work of Hahn and Novak:

"In summary, the development of white adipose tissue in both man and rat must be considered in relation to the increase in fat content, and Thus, cell size, with age. This change can explain the decreases in many enzyme activities expressed per unit of wet weight observed during postnatal development. In addition, However, some enzyme activities per unit of cytoplasmic or mitochondrial protein are also found to be changed with age and also appear to be affected by the diet (e.g., fatty acid synthesis during the suckling period when a high fat diet is fed). Such developmental changes seem much more pronounced in man than in the rat."

6.2.3 The Adipocyte as a Source and Target for Inflammation

As Rajala and Scherer state:

"Obesity is associated with an increase in TNF production in adipose tissue …. The locally elevated TNF directly interferes with proper insulin signal transduction through specific phosphorylation of critical serine residues in the insulin receptor and insulin receptor substrate 1, thereby leading to a local desensitization to insulin signaling …). In addition to local increases in TNF, a systemic increase in inflammatory markers has been shown to be associated with obesity. C reactive protein (CRP) is an unspecific acute phase reactant that serves as an excellent indicator of systemic inflammation...

Insulin resistance is not only associated with a significant increase in CRP, but a whole host of additional acute phase reactants that are elevated as well. Many of these additional factors including IL 6, 1 acid glycoprotein, and serum amyloid A (SAA) are expressed in adipose tissue... All of these proteins (with the exception of CRP) are up regulated in adipose tissue in the insulin resistant state. Increased serum IL 6 is predictive of future cardiovascular problems…. SAA can effectively compete for binding of apolipoprotein A I on high density lipoprotein particles, thereby altering trafficking of these particles ..."

Thus, the fat cells are generators of inflammatory products that place added stress upon the body.

Further work as discussed by McGillis also focuses on the immune system response to excess adipocytes. McGillis states:

"Over the last decade, the immune system and inflammatory processes have been implicated in many diseases where their involvement had not previously been appreciated. Diabetes, a serious metabolic disease, and Alzheimer's disease, a neurodegenerative disorder, are but two examples ... We also have recognized that the immune system, once considered by many to function independently of outside influence, is subject to regulatory actions of both neural and endocrine

systems…. an important piece of evidence to the growing list suggesting that WAT and its soluble products adiponectin and leptin influence immune and inflammatory functions…"

6.3 Obesity, Cancer, and the Immune System

There have been extensive studies demonstrating the impact of obesity on cancer incidence[17]. Inflammation is one of the main reasons and the generation of many reactive oxygen species is also a key driver. In addition, obesity and the related function of the excess adipocytes has been shown to inhibit effective immune system functioning. As Li et al have noted:

According to World Health Organization's 2018 report, 39% of adults aged 18 years and over were overweight (BMI≥25) in 2016, and 13% were obese (BMI≥30). Overweight and obesity are associated with increased risk of liver cancer and malignant melanoma. During obesity, pro-inflammatory adipose tissue macrophages become more abundant and hundreds of adipokines secreted by adipose tissue may regulate cancer pathogenesis.

Immune system continuously monitors cells and tissues including incipient cancer cells, overexpressed programmed death ligand 1 (PD-L1) in the tumor microenvironment engages programmed death 1 (PD-1) and subsequently triggers inhibitory signaling downstream of the T cell receptor. Agents targeting PD-1/PD-L1, such as anti-PD-1 or anti-PD-L1 monoclonal antibody, displayed impressive antitumor effects in several malignancies and are now hailed as a great breakthrough in oncology. Although overweight could be considered a tumorigenic immune-dysfunction that could be effectively reversed by anti-PD-1/PD-L1 therapy, the relationship between adipose tissue and PD-1/ PD-L1 is still ambiguous.

Cytokines are major regulators of adipose tissue metabolism. It has been shown that adipocytes can synthesize both Tumor necrosis factor (TNF-α) and several interleukins, notably IL-6. Negative impact of TNF-α on insulin sensitivity in obesity has been reported decades ago. The obesity-induced inflammatory microenvironment is a major drive of tumor progression, characterized by the presence of proinflammatory cytokines such as TNF-α. IL-6 is an important signaling molecule to affect immune system, lipid metabolism, insulin resistance, mitochondrial activity, and also promotes hepatocellular carcinoma (HCC) and melanoma progression.

Recently, TNF-α and IL-6 have also been found as regulators of PD-L1 in a variety of cells, However, whether they can regulate PD-L1 expression in HCC or melanoma is unknown. Nuclear factor κB (NF-κB) is a family of transcription factors and can be activated by TNF-α receptor. NF-κB contributes to oncogenesis in a majority of cell types including HCC. Similarly, STAT3, a transcription factor, acts downstream of various cytokines and is found to be constitutively active in a variety of cancers.

A recent study demonstrated increased hepatic pSTAT3 level in obese mice and human and identified STAT3 as a driver of HCC progression. More importantly, NF-κB and STAT3 are reported to be key mediators of PD-L1. Based on those findings above, the present study was

[17] https://www.researchgate.net/publication/265206539_Obesity_and_Type_2_Diabetes_Cause_and_Effect

designed to reveal the development of hepatocellular carcinoma in obese mice and to clarify if adipocytes regulate PD-L1 expression and the underlying mechanism….

The present study, for the first time, demonstrated that TNF-α and IL-6 secreted by adipocytes could upregulate PD-L1 level in HCC and melanoma. This may be partially involved in the role of obesity in promoting tumor progression. Considering that WAT accounts for a certain proportion in obese individuals, targeting WAT may improve metabolic environment and reduce tumorigenesis

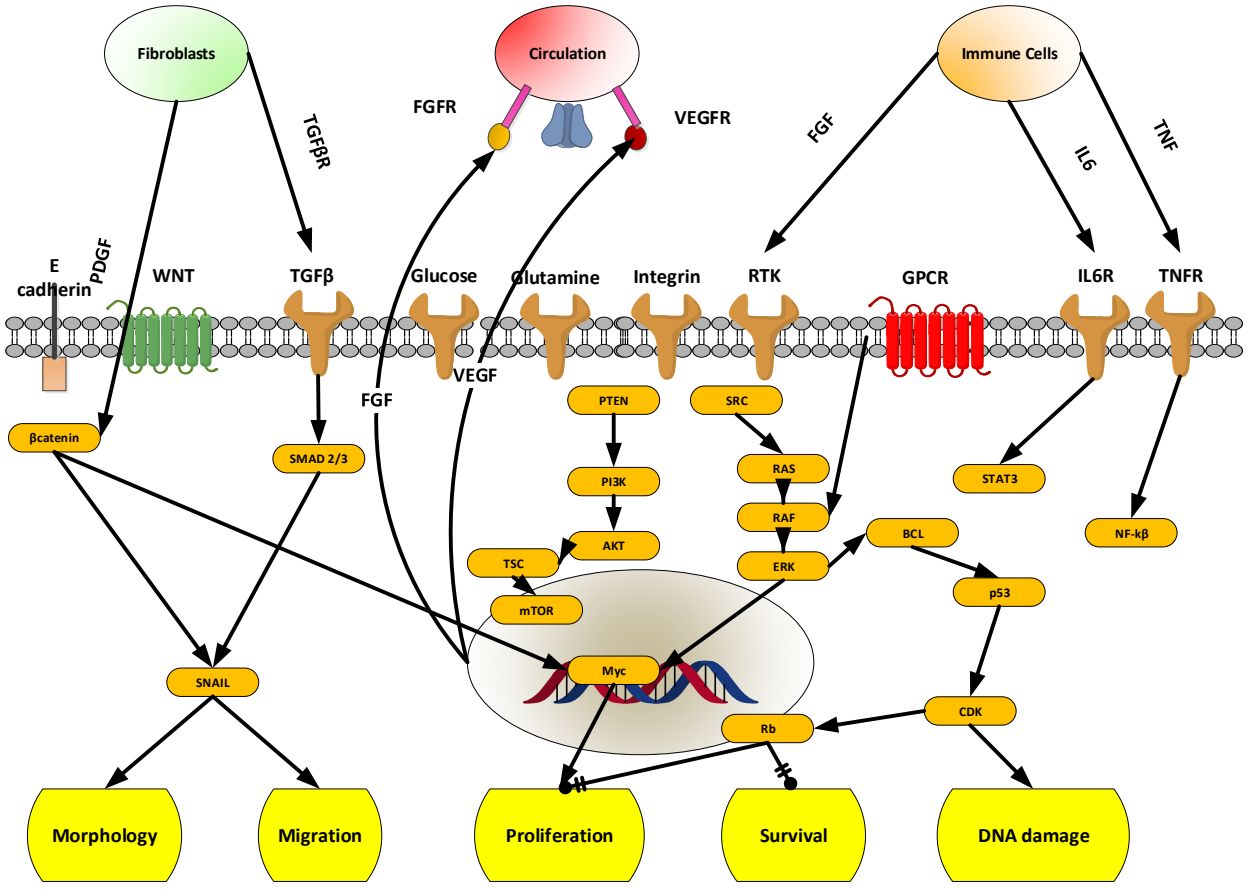
7 MACROPHAGES AND TAM

Macrophages are scavengers which go about seeking invaders in the body and then acting in such a manner to activate other immune cells. The problem is that as we have noted, these macrophages can turn on the host and inhibit their essential function of protecting the host to then protecting the invader. These macrophages are call Tumor Associated Macrophages.

7.1 OVERVIEW

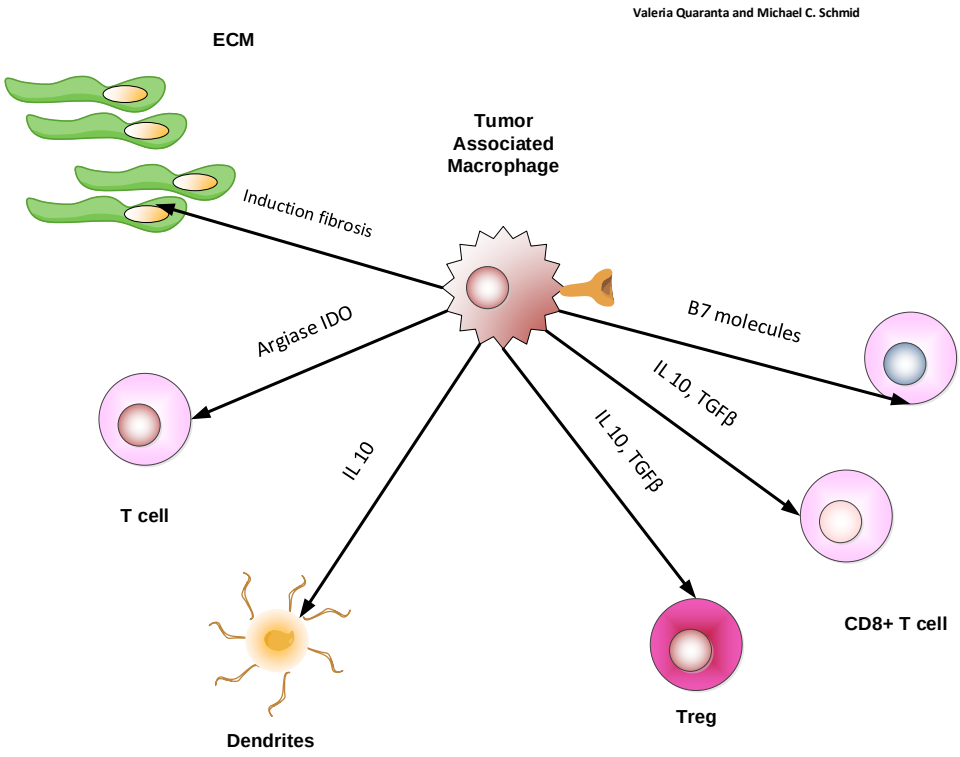
We briefly examine the tumor associated immune cells which we focused upon earlier. Again we look at macrophages, mast cells and neutrophils. As with many immune cells they can sense the state of cells they come in contact with, react to stimuli from other cells and send out stimuli to those in their environment.

The tumor micro environment, TME is complex. One may look at it as follows:



The above is a simplified attempt to demonstrate the complexity of the cell, the extracellular matrix[18], the immune system[19] and the circulatory system[20].

Let us begin with macrophages. To the beginning student of the immune system one often sees the macrophage as that wandering cell that sense invaders and then sends out signals as to their presence. In a simple sense this is the case. But then again as with all immune system elements it is always more than that.



Grivennikov et al note:

The most frequently found immune cells within the tumor microenvironment are tumor-associated macrophages (TAMs) and T cells. TAMs mostly promote tumor growth and may be obligatory for angiogenesis, invasion, and metastasis, and high TAM content generally correlates with poor prognosis.

As DeVita et al have noted[21]:

[18] https://www.researchgate.net/publication/315374581_Extracellular_Matrix_vs_Intracellular_Pathways

[19] https://www.researchgate.net/publication/314090163_Cancer_Immunotherapy_A_Systems_Approach

[20] See Cantley et al p 419 as modified.

[21] DeVita et al p 124

For example, tumor-associated macrophages (TAMs) can comprise a large proportion of tumor bulk. TAMs are often found at points of basement membrane breakdown and at the invasive front. By producing uPA, MMP7, and MMP9, TAMs help tumors degrade extracellular proteins.

The numerous growth factors that TAMs produce:

FGF, fibroblast growth factor

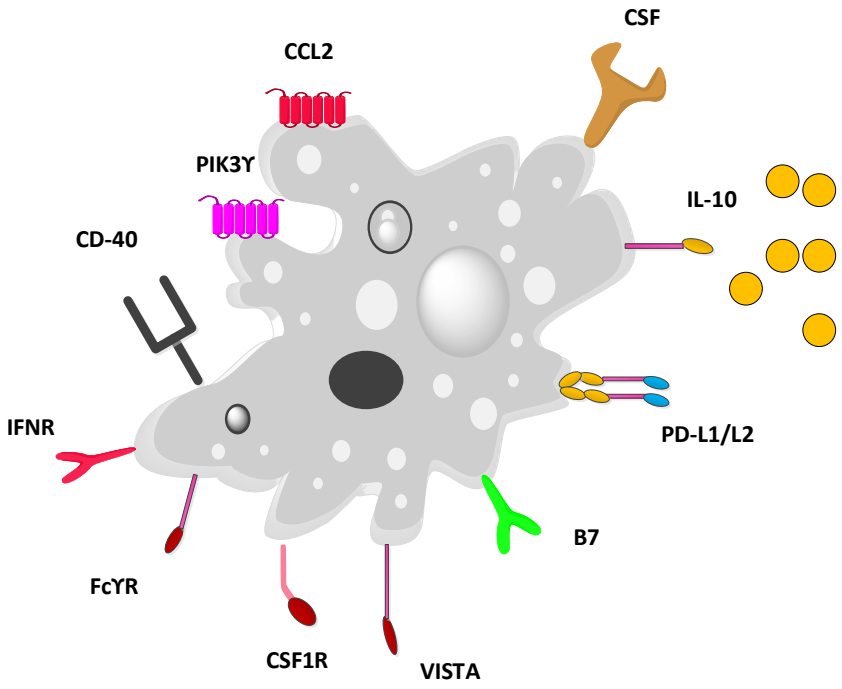
EGF, epidermal growth factor receptor ligands, and

PDGF, platelet derived growth factor, stimulates tumor cell growth and motility.

As in normal wound healing, these growth factors secreted by the TAMs or the tumors themselves activate fibroblasts.

These carcinoma-associated fibroblasts (CAFs) promote primary tumor growth by secreting stromal cell-derived factor 1 (SDF-1 or CXCL12), the ligand for CXCR4 on tumor cells. Angiogenesis is also aided by the action of CAFs through recruitment of endothelial progenitor cells by CXCL12 ***and by the action of TAMs that are recruited to areas of hypoxia to produce VEGF****. To ensure the loyalty of TAMs in promoting tumor growth, the tumor microenvironment can contain immunomodulatory factors like* ***TGF-β****, cyclooxygenase-2 (COX2),* ***CSF-1*** *(macrophage growth factor, colony-stimulating factor-1), IL-10, and IL-6, which inhibits maturation of dendritic cells and promotes TAMs that are immunosuppressed*

The TAM appears as below in terms of its receptors.



We shall examine these surface proteins in some detail as they apply to the development of a malignancy. Now as DiNardo and Ruffel note:

The presence of tumour-associated macrophages (TAMs) is generally associated with a poor prognosis in solid tumours. This has been shown in studies performed on individual tumour types using traditional immunohistochemistry techniques to quantify cellular density and in more recent analyses that infer the presence of macrophages across malignancies using gene expression profiles. These findings are consistent with the established role of macrophages in promoting multiple aspects of tumorigenesis in experimental models, from initiation through to angiogenesis and systemic dissemination.

Most relevant for patients, TAMs are known to suppress responses to standard-of-care therapeutics, including chemotherapy, irradiation and angiogenic inhibitors. Although this includes direct regulation of survival and cell death pathways in tumour cells in vivo modelling indicates that improved efficacy following macrophage depletion is often dependent upon enhanced recruitment or function of cytotoxic CD8+ T cells.

Perhaps not surprisingly, macrophage antagonists demonstrate combinatorial efficacy when combined with immunotherapy, including checkpoint blockade. Clinical trials examining these combinations are now ongoing. In this Review, we discuss how macrophages are induced into becoming immunosuppressive, the mechanisms by which they suppress antitumour immunity and how this information is being utilized to develop therapeutics and design clinical trials.

From Wilke et al in Curiel we have:

TAMs (tumor associated macrophages) form the major APC subset (by number) in solid human epithelial cancers. Several years ago, our group discovered that both tumor cells and microenvironmental macrophages in ovarian cancer expressed CCL22, a chemokine instrumental in attracting Tregs to the tumor environment.

Interestingly, because the presence of Tregs predicts poorer survival and is associated with a high death hazard in ovarian cancer patients, TAMs may contribute to their prognoses. Indeed, we subsequently demonstrated that although they are highly B7-H4 positive, ovarian cancer cells do not directly mediate antitumor T cell suppression. However, B7-H4+ macrophages from the human ovarian tumor microenvironment are powerful suppressors of tumor-associated antigen-specific T cell immunity. B7-H4 blockade restored the stimulatory capacity of macrophages and mediated ovarian tumor regression in vivo in NOD/SCID mice. Both IL-10 and IL-6, often found in high concentrations in the tumor environment, can induce B7-H4 expression on macrophages.

Contrastingly, two cytokines minimally expressed in the same environment—GM-CSF and IL-4—inhibit B7-H4 expression. Interestingly, forced expression of B7-H4 in macrophages from healthy donors conferred a suppressive phenotype on the cells. As for the prognostic significance of B7-H4+ macrophages in ovarian cancer, we documented an inverse relationship between the intensity of B7-H4 expression on macrophages and patient survival. Importantly, Tregs, typically predictors of poor prognoses in cancer patients, could induce B7-H4 expression on myeloid APCs (including macrophages) and were positively associated with B7-H4+ macrophage presence in ovarian tumors.

A later observation of Wan and colleagues showed that the mean density of TAMs is significantly higher in ovarian cancer than in benign ovarian lesions and that the average 5-year survival rate in patients with low densities of TAM was significantly higher than in patients with larger TAM populations, agreeing well with our observations. Multivariate analysis demonstrated that TAM infiltration status serves as an independent negative predictor for overall survival of patients with ovarian cancer. The presence of CCL17+ or CCL22+ cells in CD14+ monocytes and macrophages within gastric tumors correlated directly with Treg cell presence. Tregs were also shown to migrate toward CCL17 and CCL22

7.2 TAM FUNCTIONING

Let us first examine the bifurcation of the macrophages. As Kundu and Surh note:

Tumor-associated macrophages, mast cells and neutrophils play an important role in tumor angiogenesis by secreting VEGF, IL-8, TNFa, MMPs and other factors that increase vascular permeability.

Thus, chronic inflammation-driven tumor angiogenesis and a sustained 'inflammation-cancer-inflammation' loop proves Dvorak's early proposition that tumors are wounds that never heal. The role of various proinflammatory mediators in tumor angiogenesis will be discussed further.

Poh and Ernst note a more differentiated characterization of M1 and M2, separating M2 into four subsets as follows:

Tumor-associated macrophage heterogeneity is not only dependent on the nature of their monocytic precursor, but also on their functional diversity. To coordinate complex processes to promote immunity, while also minimizing damage to tissues where these responses occur, macrophages can reversibly alter their endotype in response to environmental cues.

These environmental cues include stimuli derived from pathogens, parenchymal, and immune cells, as well as the extracellular matrix. Similar to the Th1/Th2 T-cell dichotomy, macrophages may be broadly classified into two groups, referred to as:

(i) ***"classically activated M1" (CAM) or***

(ii) ***"alternatively activated M2" (AAM) endotypes.***

Much our understanding of macrophage polarization has relied on ***in vitro techniques****, whereby macrophages are stimulated with M1- or M2-polarizing signals.*

(i) ***For M1 this typically involves stimulation with IFNγ or lipopolysaccharide (LPS),***

(ii) ***while M2 polarization usually involves stimulation with IL4 or IL13.***

Changes in gene expression, cell-surface markers and signaling pathways have subsequently been used to distinguish the various activation states, and the contribution of some of these factors in mediating CAM/AAM characteristics has been validated in genetically engineered mouse models.

However, given the heterogeneity of tissues, macrophage polarization should be regarded as a complex process that occurs over a continuum. The current classification of CAM or M1 macrophages is in part based on their response to stimulation with bacterial LPS, TNFα, and/or IFNγ. TNFα is produced by antigen presenting cells upon recognition of pathogenic signals, while IFNγ is produced by innate and adaptive immune cells such as natural killer (NK) and Th1 cells. Once activated, CAMs secrete pro-inflammatory cytokines (IL1, IL6, and TNFα) and effector molecules (including reactive nitrogen intermediates) and express chemokines such as CXCL9 and CXCL0.

These molecules exert and amplify antimicrobial and tumoricidal activities alongside increased Th1 adaptive immune responses through enhanced antigen presentation. Because these cytokines play an important role in immune defense, their inappropriate release can result in chronic inflammation and extensive tissue damage.

Alternatively, activated M2 macrophages are broadly characterized by their anti-inflammatory and wound-healing endotype*. While these functional outputs are important for the maintenance of tissue homeostasis, aberrant AAM activation can trigger allergic reactions, promote tumor growth, and delay immune responses toward pathogens.*

Among the most important activators of AAMs are IL4, IL10, and IL13; However, several other stimuli and signaling pathways can also induce AAM polarization.

Thus, AAMs can be further divided into M2a, M2b, M2c, and M2d. The M2a subtype is stimulated in response to IL4, IL13, as well as fungal and helminth infections.

M2a macrophages express high levels of mannose receptor (CD206) and secrete large amounts of pro-fibrotic factors including fibronectin, insulin-like growth factor and TGFβ, which are all involved in wound healing and tissue repair.

M2b macrophages are stimulated by immune complexes and bacterial LPS and exhibit upregulated expression of CD206 and the MER receptor tyrosine kinase. *They primarily produce IL10, IL1β, IL6, and TNFα, which exert anti-inflammatory effects.*

M2c macrophages *are activated by IL10, TGFβ, and glucocorticoids and are also generally thought to be anti-inflammatory in nature. Finally, differentiation of*

M2d macrophages *occurs in response to co-stimulation with* ***TLR ligands and adenosine.*** *M2d macrophages express low levels of CD206 but are high producers of* ***IL10 and VEGF****. In light of these findings, it is now appreciated that the "AAM" terminology encompasses a functionally diverse group of macrophages that share the functional outputs of tumor progression by stimulating immunosuppression and angiogenesis.*

We summarize the above in the following table.

Type	***Activated by***	***Produce***
M1	*stimulation with IFNγ or lipopolysaccharide (LPS)*	
M2a	*stimulation with IL4 or IL13*	*mannose receptor (CD206) and secrete large amounts of pro-fibrotic factors including fibronectin, insulin-like growth factor and TGFβ,*
M2b	*by immune complexes and bacterial LPS*	*upregulated expression of CD206 and the MER receptor tyrosine kinase.*
M2c	*activated by IL10, TGFβ, and glucocorticoids*	
M2d	*co-stimulation with TLR ligands and adenosine*	*CD206 but are high producers of IL10 and VEGF.*

From Laviron and Boissonnas we have an interesting reconfiguration of this M1 and M2 fabric. They authors present a somewhat alternative view as follows:

Tumor-associated macrophages (TAM) represent a major component of the tumor microenvironment (TME) that has been extensively studied in the past decades. They play a major role in tumor growth, metastatic dissemination, and therapy failure. Countless reports have described that TAMs can promote angiogenesis, inhibit the anti-tumor immune response, in particular T-cell-mediated cytotoxicity, support tumor growth, and secrete different factors involved in extracellular matrix (ECM) remodeling Thus, facilitating tumor cell motility and intravasation. High TAM infiltration is generally correlated with poor outcomes in several types of cancer, such as breast, ovarian, and lung cancer.

However, in some indications TAM can be associated with enhanced anti-tumor immunity. Although macrophages were originally described as arising exclusively from circulating monocyte precursors, it was shown in the recent years that several organs harbor embryonic-derived populations of ***resident macrophages (ResMac)*** *that maintain and self-renew throughout adulthood.*

This new concept ***challenges the dogma of TAM origin and questions their relative function. TAM subsets were originally classified as tumoricidal vs. tumor-promoting, often referred as M1/M2 macrophages,*** *based on the expression of specific markers. However, the wide diversity of TAM cannot be covered by this nomenclature and many subsets express overlapping markers of the M1/M2 polarization.*

Whether TAM heterogeneity originates from their high plasticity or rather from independent specific lineages giving rise to multiple populations is still unclear. Although cellular ontogeny can recapitulate parts of the heterogeneity, it appears that environmental cues are also major determinants in cell education. Macrophage diversity would then be the result not only of ontogeny but also of niche- specific signaling events of tumor immunity.

One can Thus, wonder whether the origin of TAM dictates their role in tumor development and is associated with various functions. This represent a key issue for anti-cancer therapies as these subsets might be differentially targeted regarding their role in tumor development. …

Although the precise origin of ResMac is still under debate, fate-mapping models highlighted a differential origin of tissue macrophages deriving either from an embryonic precursor (yolk sac, fetal liver) or a monocyte precursor from adult hematopoiesis origin.

These precursors seed the tissues in different waves during development and adulthood giving rise to different ResMac*. The dynamics of these waves vary between organs, age, and macrophage subsets.*

In some organs, such as the brain, the lung and the liver,

(i) some ***embryonic-derived ResMac (named here EmD-ResMac)*** *maintain by self-renewal in adults whereas in the gut, the skin, the heart, and the pancreas*

(ii) most subsets are progressively replaced through the dierentiation of monocyte precursors from adult hematopoiesis into ***monocyte-derived ResMac (named here MoD-ResMac****) with different turnover rates.*

The ability of newly recruited macrophages to self-maintain in the tissue and become a ResMac per se is proposed to be tightly regulated by space availability and competition for growth factors in the niche. This turnover appears to be variable among subsets in a given organ and could be induced by exposure to homeostatic environmental cues (e.g., mechanical, metabolic) specific of distinct sub-tissular regions.

In the gut, long-lived macrophages with precise sub-tissular localization are key regulators of physiological functions. In the lungs, alveolar macrophages (AM) originate almost exclusively from yolk-sac derived macrophages and self-maintain throughout adulthood, whereas lung interstitial macrophages follow a more complex regulation, unveiling further heterogeneity in this subset. While some of these interstitial macrophages have an embryonic origin , others differentiate from distinct monocyte precursors according to the sub-tissular niche they colonize, Thus, becoming the dominant population during adulthood. …

The common characterization of TAM subsets relies on the M1/M2 polarization model induced by different in vitro stimuli. This model rapidly finds limitation in complex environments (in vivo) in which M1 and M2 stimuli can be present and generate very dynamic microanatomical niches.

Tumors should be considered as an evolving tissue in which space availability and growth factors expression are changing over time and where inflammatory signals are generated by the loss of tissue integrity and immune cell infiltration.

It is Thus, not surprising to find a wide range of activation profiles in the TME. No typical M1/M2-associated marker defined one or the other TAM subset in lung unveiling heterogeneity among each subset.

No direct link between TAM origin and the commonly described pro- or anti-tumor profile could be achieved in this study. One could expect that macrophage ontogeny and their anatomic localization define specific niches dictating their polarization toward a specific phenotype and function.

Thus, one may conclude that the TAMs are of varying types activating and being activated in a multiplicity of ways. As Monteran and Erez have noted:

Macrophages can be classified according to their functional differentiation state and immunological responses:

M1-like macrophages are involved in the response of type I T helper cells (Th1), they are activated by interferon gamma (IFNγ) and engagement of Toll-like receptors (TLRs) and characterized by production of pro-inflammatory molecules, nitric oxide (NO) and reactive oxygen species (ROS).

M2-like macrophages, which are in general protumorigenic, are involved in Th2-type immune responses, wound healing and tissue repair, activated by IL-4 and IL- 13, and characterized by promotion of angiogenesis and secretion of immune suppressive factors that inhibit killing by cytotoxic T cells.

This classification However, is not dichotomous, and different macrophage subtypes may share multiple features. Functional differentiation of TAMs in the tumor microenvironment is affected by many factors. Recently, CAFs are emerging as novel effector cells in TAM differentiation toward an immunosuppressive phenotype, in addition to their role in monocyte recruitment. CAF-derived Chi3L1 was shown to be important for both recruitment and functional differentiation of bone marrow-derived macrophages in a mouse model of breast cancer: Genetic targeting of Chi3L1 expression in fibroblasts attenuated macrophage recruitment and their reprogramming to an M2-like phenotype, and promoted a Th1 phenotype in the tumor microenvironment.

Prostate CAFs were shown to mediate both the recruitment and the M2-like differentiation of monocytes via SDF1. A similar finding was demonstrated in an ex-vivo model of oral squamous cell carcinoma. CAFs isolated from human tumors, instigated an M2-like phenotype in patient-derived CD14+ myeloid cells (manifested by production of ARG1, IL-10 and TGF-β), which in turn potently suppressed the proliferation of autologous T cells.

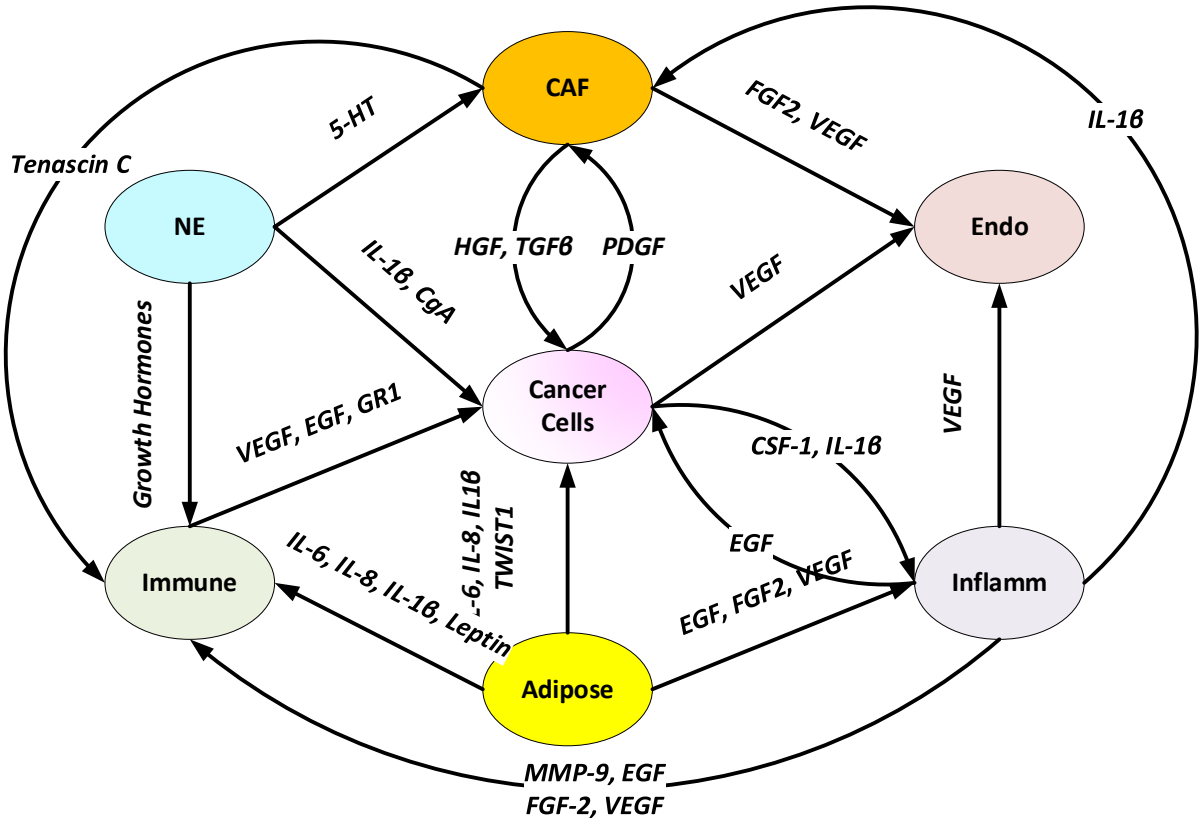
However, the underlying CAF-derived factors that mediated M2-like differentiation were not identified. In this context, it is important to note that while TAMs and CAFs are both central players in the tumor microenvironment, their reciprocal interactions in cancer are not well characterized, and the main focus in the literature is on the effects of macrophages on fibroblasts. Future studies are required to further elucidate the contribution of CAF-derived signaling to the diverse functions of macrophages in the TME

We can also see these different TAMs in a variety of other malignancies.

8 CANCER ASSOCIATE FIBROBLASTS, THE IMMUNE SYSTEM AND ECM SIGNALING

As we had noted earlier, the cancer cells are not entities unto themselves. They function in a highly holistic environment, communications with multiple classes of other cells. We show this in an example below based upon the work of Wang et al. Cancers are systems, complex communicating interactive systems. The external communicating links shown below are but one significant example. Within the cancer cell itself is also a set of communicating links but the external environment is often what protects the cancer cells from effective therapeutics.

The diagram below shows the interfaces between the cancer cell and the other cellular entities within the TME. Note that the cancer cells can reach out, directly or otherwise, to all other cells and in so doing create a highly protective environment. This diagram demonstrates cells and signalling mechanisms. The signalling mechanisms may also present therapeutic targets.



8.1 CAF, FIBROBLASTS

Fibroblasts can be altered in a synergistic manner by the cancer cells and turned into Cancer Associated Fibroblasts, CAF. Thus, the TME can be seen to be filled with the CAF cells.

The CAF are those fibroblasts which have been effectively captured and used by the cancer cell to both protect it and to allow it to propagate. As Cirri et al have noted:

Fibroblasts are the most abundant cell type in connective tissues and form the structural framework of tissues through their secretion of ECM components. Quiescent fibroblasts undergo activation during wound healing and fibrosis, both conditions sharing the requirement for tissue remodelling, and become myofibroblasts (MFs), as originally described by Giulio Gabbiani in 1971. MFs acquire contractile stress fibers, de novo express α-smooth muscle actin (α -SMA) and the ED-A splice variant of fibronectin, and form cell–cell contacts through gap junctions .

Upon completion of the wound healing process, activated fibroblasts undergo a particular type of programmed cell death, called nemosis, and are removed by the granulation tissue. Considering that "tumors are wounds that do not heal",

CAFs share some similarities with MFs, including expression of SMA and ED-A fibronectin, but greatly differ for their duration (they are not removed by apoptosis) and their activation is not reversible. CAFs are the most prominent cell type within the tumor stroma of many cancers, most notably breast, prostate and pancreatic carcinoma. Recent studies underscore several subpopulations of stromal fibroblasts within different tumors.

These populations share some properties collectively leading to their "activation state", although their expression of acknowledged activation markers is only partial. The main activation markers are α-SMA and fibroblast specific protein (FSP), although platelet-derived growth factor (PDGF) receptors-β and fibroblast activation protein (FAP) have been found overexpressed in stromal fibroblasts of solid tumors. Beside these molecular markers of fibroblasts activation, some other proteins expressed by stromal fibroblasts are recognised to have a prognostic value for solid tumors. In particular, a poor prognosis has been associated with expression in CAFs of the hypoxia marker carbonic anidrase IX in human lung adenocarcinoma, or periostin in cholangiocarcinoma, or p53 tumor suppressor in ductal carcinoma.

On the contrary expression in CAFs of Caveolin-1, PTEN or podoplanin correlates with a favourable prognosis for several carcinoma. Indeed, recent studies have reported a tumor promoter effect of p53 inactivation in the stromal fibroblasts, as well as that genetic inactivation of PTEN in CAFs accelerates both onset and progression of breast carcinoma. This large heterogeneity in marker expression for CAFs originating from different tumors may be explained by their possible diverse origin. Indeed, CAFs are variously reported to stem from resident local fibroblasts, bone marrow derived progenitor cells or trans-differentiating epithelial/endothelial cells through epigenetic transitions.

One of the driving issues is that one should be examining the cancer as a complex entity and one which includes not only the basic organ transformed cells but the entire environment. As Fabian and Storkusnote (in Kalinski) note:

Cancer-associated fibroblasts (CAFs) are spindle shaped cells embedded within the extracellular matrix (ECM) that originate from resident fibroblasts and bone marrow-derived mesenchymal precursors. They are phenotypically and functionally distinct from normal fibroblasts and comprise a significant component of the tumor stroma. Although technically not part of the blood vessels, cancer-associated fibroblasts still play a major role in angiogenesis and in the promotion of the tumor blood vessel formation, mostly by producing pro-angiogenic factors.

Although cancer cells themselves can release VEGF, the principal source of VEGF in the tumor microenvironment is the CAFs. In cancers such as pancreatic cancer and breast cancer, CAFs produce stromal-derived factor 1 (SDF-1) or CXCL12, which contribute to tumor vascularization by recruiting endothelial precursor cells from the bone marrow. CAFs could also indirectly promote tumor angiogenesis by secreting chemokines like CXCL1 and CXCL2 to recruit pro-angiogenic macrophages and neutrophils into the tumor microenvironment. Furthermore, CAFs release matrix metalloproteinases that degrade the ECM, Thus, spatially accommodating the growing blood vessel and, at the same time, releasing VEGF previously sequestered in the ECM.

The above demonstrates the facilitating mechanisms used by and available to the CAF in the process of malignant propagation. The CAF also possess a broad spectrum of forms and presentations. As Junttila1 & de Sauvage note:

Fibroblasts are an abundant mesenchyme-derived cell type that maintain the structural framework in tissues. Quiescent fibroblasts differentially respond to damage, such as wounding, and become activated to support repair. Although normal fibroblasts typically suppress tumour formation, cancer-associated fibroblasts (CAFs) can significantly promote tumorigenesis4.

Compared with normal tissue fibroblasts, CAFs have increased proliferation, enhanced extracellular matrix production and unique cytokine secretion (for example, stromal cell-derived factor 1, SDF1; vascular endothelial growth factor, VEGF; platelet-derived growth factor, PDGF; and hepatocyte growth factor, HGF). Other mesenchyme-derived cell types, such as adipocytes, can also contribute to tumour growth and progression.

Phenotypic and functional heterogeneity occurs in healthy fibroblasts and CAFs. Differences in fibroblast behaviour and response lead to extensive tissue remodelling mediated by augmented expression of proteolytic enzymes (for example, matrix metalloproteinases), deposition of extracellular matrix and pathogenic angiogenesis by liberating proangiogenic factors within the matrix. Heterogeneity may be attributable to unique damage signals to which fibroblasts are exposed or possibly to their origin.

Significant cell plasticity also exists within this cell population, as both mesenchymal-to-epithelial transitions, and epithelial-tomesenchymal transitions are known to occur, further

enhancing stromal heterogeneity. Although extensive dissection of stromal fibroblast intra- and intertumoral heterogeneity (variation between tumours) is impaired by the lack of specific markers, CAFs within tumours are clinically relevant. For example, the abundance of stromal cells correlates with poor prognosis for several forms of cancer, including breast and pancreatic cancer. Elevated expression of matrix metalloproteinases correlates with increased aggressiveness and poor prognosis in certain cancers. A significant link between increased adipose tissue and cancer risk has also been demonstrated, although the mechanisms are still being elucidated.

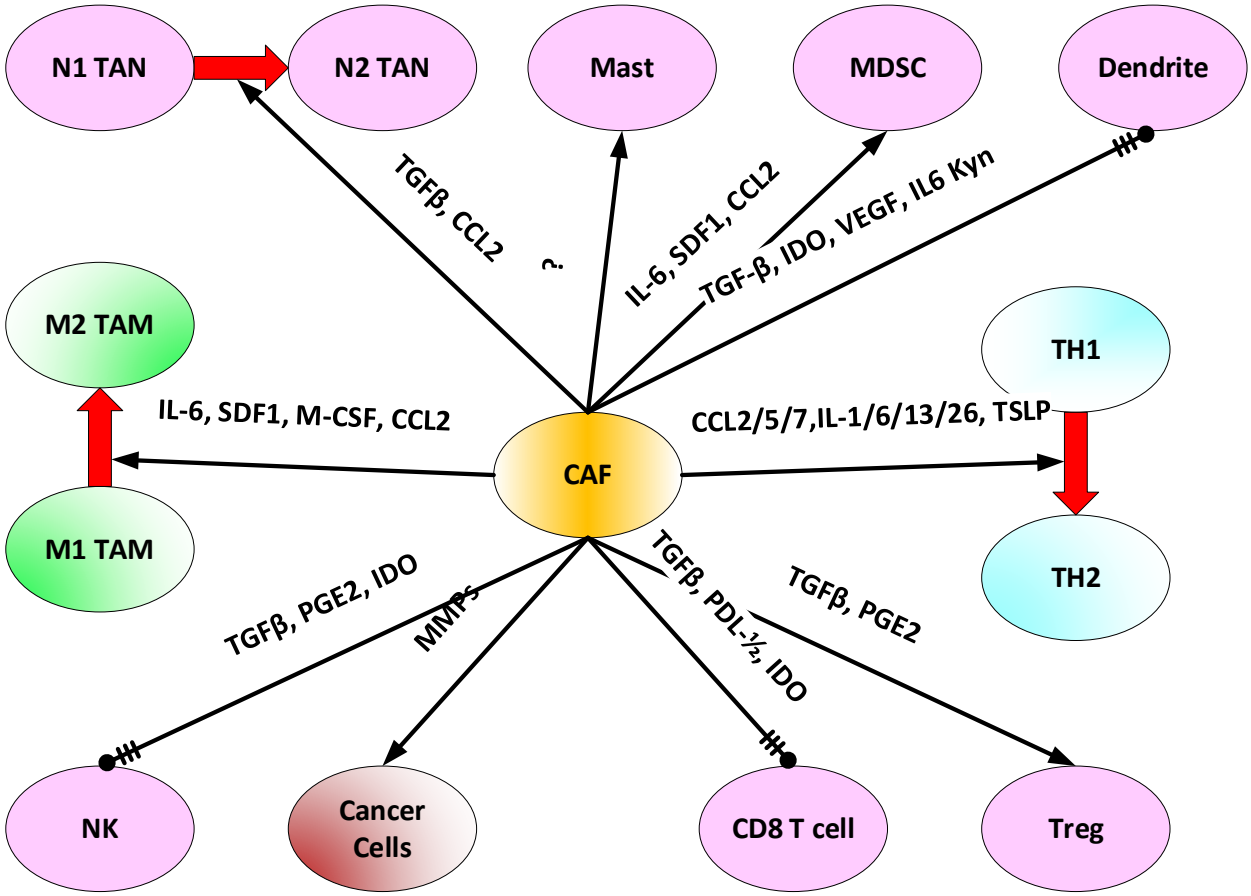
8.2 THE IMMUNE INTERFACE

We often think of the immune system as a means to attack cancer cells[22]. However, the immune system can also become a protector of the cancer cells. From the work of Ziani et al we show a modified version of their example wherein the CAF can create precancerous environments by blocking good cells and by transforming normally protective cells into cells protective of the cancer cells themselves. We have previously discussed tumor associated immune cells, TAI, which include the Tumor Associated Macrophages, TAMs[23].

The Figure below is detailed presentation of all of the currently understood interfaces. The M1/M2 transitions are significant and we have discussed them. The NK blockage is extremely concerning for a variety of cancers. If the CAF can block NK early on then the first order of defense is depleted. We believe that such may be the case in HGPIN in the prostate. Likewise, the same may be the case for CIS in certain melanoma presentations. In a broad sense the CAF spans all the major immune response mechanisms. The links as demonstrated may also be putative therapeutic targets.

[22] https://www.researchgate.net/publication/314090163_Cancer_Immunotherapy_A_Systems_Approach

[23] https://www.researchgate.net/publication/336116071_Tumor_Associated_Immune_Cells_On_the_one_hand_and_on_the_other_hand



The above is a significant description of how just one element of the TME, the fibroblast, can negatively impact the overall immune protection, and in fact turn the immune system against its host. The activation of M1/M2 transitions alone facilitates the TAM process. It also blocks any attempt to inhibit the ability to use immunotherapeutic agents. Perhaps it is via this above process that we find the source of delimiting immunotherapeutic agents.

As Ziani et al note:

It is now well admitted that tumor progression and metastasis formation do not only depend on cancer cell genetic and epigenetic defects but are also controlled by the tumor microenvironment (TME). The TME or stroma is composed of cells from endothelial, mesenchymal, and hematopoietic origins embedded in a complex extracellular matrix (ECM), which enter into a dynamic crosstalk with tumor cells, suitable for tumor growth. Consequently, different elements such as angiogenesis, hypoxia, ECM remodeling, interstitial pressure, metabolism changes have received recent attention as key determinants of the TME modifying cancer cell behavior and disease progression, with potential clinical applications.

Moreover, the TME is also clearly involved in shaping the cellular fate of tumor-infiltrating lymphocytes and the efficacy of the anti-tumor immune response. Indeed, during tumor

progression, tumor cells proliferate under adverse host conditions and use several survival strategies to block the action of key regulators/effectors of the immune response and to circumvent anti-tumor defenses. Besides the several known classical strategies used by tumor cells to escape immune surveillance (such as down regulation of antigen expression, resistance to cell-mediated lysis or expression/secretion of immunosuppressive molecules), it should be noted that tumor cell evasion from immunosurveillance is also under the control of the TME complexity.

The ability of tumors to orchestrate an immunosuppressive microenvironment is dependent on several mechanisms ultimately leading to the inhibition of various immune effector cells [such as cytotoxic T cell (CTL) or natural killer (NK) cells] or to the recruitment and stimulation in the TME of immunosuppressive cells [such as regulatory T cells (Tregs), type II macrophages or myeloid derived suppressor cells (MDSCs)]. In particular, among the stromal cells, activated fibroblasts that share similarities with fibroblasts stimulated by acute or chronic inflammatory signals, activated during a wound healing process and observed during tissue fibrosis, also known as myofibroblasts, play a critical role in the complex process of tumor cell-stroma interaction and have emerged as important regulators of the anti-tumor immune response

They continue:

Based on the immunomodulatory secretome mentioned above, CAFs might also interfere with the adaptive anti-tumor immune response at different levels, leading to a disruption of T cell function in the TME (Figure 2). In the TME, dendritic cells (DCs), the most important antigen-presenting cell population, have a pivotal role for the activation of T cell-mediated anti-tumor immunity (103). DC biology can potentially be affected by the CAF secretome in several ways.

In particular, CAF-derived TGF-β can affect DC function.

In response to TGF-β, DCs downregulate the expression of MHC class II molecules and of the co-stimulatory molecules CD40, CD80, and CD86, which are necessary for efficient antigen presentation, and of TNF-α, IFN-γ, and IL-12, that promote T cell recruitment and survival.

The resulting immature or tolerogenic DCs alter CD8+ cytotoxic T cell activation and the TH1 polarization of CD4+ helper T (TH) cell populations and also promote the formation of CD4+FoxP3+ Treg cells that potently inhibit the function of other T cells.

CAFs can also secrete IL-6 and could affect DC functions through this way. Indeed, IL-6-mediated activation of the STAT3 pathway has been involved in the alteration of the DC maturation, disabling T cell activation and inducing T cell anergy and immune tolerance.

Fibroblast-produced IL-6 was also reported to favor the emergence of TAMs from monocytes at the expense of DCs.

Expression of tryptophan 2,3-dioxygenase (TDO2) by CAFs isolated from lung cancer also promotes tryptophan degradation in kynurenines (Kyn) that inhibits DCs differentiation and functions. Finally, CAF-derived VEGF, in addition to its proangiogenic effect, has multiple

immunoregulatory roles. In particular, VEGF inhibits DC generation and maturation, notably by reducing their MHC class II expression and their ability to take up antigens.

The role of ***CAFs in regulating T cell activity*** *and function in the TME has also been suggested by several studies.* ***As mentioned earlier, CAFs can be an important source of TGF-β in the TME, which may act on both CD8+ and CD4+ T cells.***

For example, TGF-β promotes cell death of effector CD8+ T cells by inhibiting expression of the pro-survival protein Bcl-2. TGF-β also directly alters cytotoxic CD8+ T cell function by inhibiting the expression of key genes involved in their cytototoxic activity, including perforin, granzymes A and B, Fas ligand, and IFN-γ. Furthermore, CAFs could also impair T cell proliferation and effector function through other mechanisms, notably depending on their production of metabolic reprogramming factors.

The secretion by CAFs of IDO1, an immuno-regulatory enzyme, might contribute to immuno-suppression, tolerance, and tumor escape by catabolizing tryptophan degradation into kynurenines (Kyn), creating an immunosuppressive TME resulting in T-cell anergy and apoptosis through depletion of tryptophan and accumulation of immunosuppressive tryptophan catabolites. *…*

9 PROSTATE CANCER: AN EXAMPLE

Prostate cancer is a common cancer in men, and in many cases one of the most common[24]. It typically is a cancer associated with age and for many men the progression can be a slow but ongoing process. For some, However, the malignancy can be highly aggressive. The distinctions in aggressiveness is yet to be fully explained.

The typical PCa histologically appears as a slowly randomizing of the prostate glands, with an initial inflammatory stage withing individual glands, then the appearance of somewhat disorganized glands and finally a totally disorganized mass of unstructured prostate cells.

9.1 PROSTATE CANCER DYNAMICS

Let us begin with some basics on PCa dynamics. As Josson et al noted:

In summary, our study demonstrated the oncogenic roles of stromal-derived miR-409 that is capable of promoting tumor growth and EMT of adjacent tumor epithelia in vivo. The transfer of miR-409 via EVs from prostate stromal fibroblasts to cancer epithelium could result in the repression of tumor suppressors, induction of EMT and the promotion of the growth and survival of prostate epithelium. Therefore, miR-409 may be a new therapeutic target for the treatment of both benign and malignant growth of the prostate gland and to break the vicious stromal–epithelial interactions that contributes to the tumor heterogeneity in the tumor microenvironment

We have discussed the miRNA possibilities at length. This means we have a broad set of dynamics in the interplay between the cancer cells and the TME.

9.2 CAF AND PCA

As Doldi et al have noted:

Tumor microenvironment coevolves with and simultaneously sustains cancer progression. In prostate carcinoma (PCa), cancer associated fibroblasts (CAF) have been shown to fuel tumor development and metastasis by mutually interacting with tumor cells. Molecular mechanisms leading to activation of CAFs from tissue-resident fibroblasts, circulating bone marrow-derived fibroblast progenitors or mesenchymal stem cells are largely unknown. Through integrated gene and microRNA expression profiling, we showed that PCa-derived CAF transcriptome strictly resembles that of normal fibroblasts stimulated in vitro with interleukin-6 (IL6), Thus, proving evidence, for the first time, that the cytokine is able per se to induce most of the transcriptional changes characteristic of patient-derived CAFs.

Comparison with publicly available datasets, However, suggested that prostate CAFs may be alternatively characterized by IL6 and TGFβ-related signatures, indicating that either signal, depending on the context, may concur to fibroblast activation. Our analyses also highlighted novel pathways potentially relevant for induction of a reactive stroma. In addition, we revealed a

[24] https://www.researchgate.net/publication/264960277_Prostate_Cancer_A_Systems_Approach

role for muscle-specific miR-133b as a soluble factor secreted by activated fibroblasts to support paracrine activation of non-activated fibroblasts or promote tumor progression.

They continue:

It is widely accepted that solid tumors are heterogeneous and complex systems consisting of neoplastic cells and untransformed stroma components. Such reactivestroma is a mixture of immune cells, endothelial cells and CAFs exhibiting activated phenotypes. CAFs are the predominant cell population in PCa microenvironment and are characterized by a phenotype reminiscent to that of fibroblasts involved in wound repair events.

Reciprocal interaction between cancer cells and CAFs influences each step of tumor development, growth and metastasis, mainly through the release of soluble growth factors. In the context of PCa, we have previously shown that CAFs, through secretion of MMPs, elicit EMT and achievement of stem cell traits in cancer cells, as well as enhancement of tumor growth and development of spontaneous metastases.

We also reported that CAFs induce a Rac1b/ cycloxygenase-2-mediated release of reactive oxygen species in carcinoma cells, which activates nuclear factor- κB and HIF-1 [17], and ultimately results in repression of miR-205 transcription. Inhibition of miR-205 function relieves a brake on its target genes, including E-cadherin transcriptional repressors, Thus, leading to establishment of EMT and enhancement of metastasis.

The other way around, tumor cells per se contribute to the formation of a reactive stroma. In this regard, we showed that PCa cells can activate normal fibroblasts to a phenotype reminiscent to that of patient-derived CAFs through the secretion of soluble factors, including IL6. In fact, stimulation of HPFs with the medium of castration-resistant PCa cells was sufficient to increase α-SMA and FAP expression and to promote the acquisition of tumor-promoting properties, a phenotype abrogated by the administration of an anti-IL6 antibody.

In the present study, we comparatively analyzed gene expression profiles of CAFs and HPFs obtained from radical prostatectomies to contribute to the understanding of the biology and signaling mechanisms involved in reactive stroma formation. Previous studies suggest that, compared to normal fibroblasts, CAFs found in tumors are characterized by enhanced collagen synthesis, secretion of growth factors and ECM modulators, and the activation of unique expression programs.

9.3 Other Interactions

As Huang et al note:

Prostate cancer stem cells (PCSCs) play a critical role in prostate cancer progression and metastasis, which remains an obstacle for successful prostate cancer treatment. Tumor-associated macrophages (TAMs) are the most abundant immune cell population within the tumor microenvironment (TME). Systematic investigation of the interaction and network signaling between PCSCs and TAMs may help in searching for the critical target to suppress PCSCs and

metastasis. Herein, we demonstrated that TAMs-secreted CCL5 could significantly promote the migration, invasion, epithelial–mesenchymal transition (EMT) of prostate cancer cells as well as the self-renewal of PCSCs in vitro. QPCR screening validated STAT3 as the most significant response gene in prostate cancer cells following CCL5 treatment. RNA-sequencing and mechanistic explorations further revealed that CCL5 could promote PCSCs self-renewal and prostate cancer metastasis via activating the β-catenin/STAT3 signaling.

Notably, CCL5 knockdown in TAMs not only significantly suppressed prostate cancer xenografts growth and bone metastasis but also inhibited the self-renewal and tumorigenicity of PCSCs in vivo. Finally, clinical investigations and bioinformatic analysis suggested that high CCL5 expression was significantly correlated with high Gleason grade, poor prognosis, metastasis as well as increased PCSCs activity in prostate cancer patients. Taken together, TAMs/CCL5 could promote PCSCs self-renewal and prostate cancer metastasis via activating β-catenin/STAT3 signaling. This study provides a novel rationale for developing TAMs/CCL5 as a potential molecular target for PCSCs elimination and metastatic prostate cancer prevention

As Noh et al have noted:

Although prostate cancer is clinically manageable during the early stages of progression, metastatic progression severely compromises the prognosis and leads to mortality. Constitutive activation of STAT3 has been connected to prostate cancer malignancy, and abolishing the STAT3 activity may diminish tumor growth and metastasis.

However, its suppressor genes and pathways have not been well established. In this study, we show that promyelocytic leukemia zinc finger (PLZF) has a putative tumor-suppressor function in prostate cancer by inhibiting phosphorylation of STAT3. Compared with a benign prostate, high-grade prostate cancer patient tissue was negatively correlated with PLZF expression. PLZF depletion accelerated proliferation and survival, migration, and invasion in human prostate cancer cells. Mechanistically, we demonstrated a novel role of PLZF as the transcriptional regulator of the tyrosine phosphatase SHP-1 that inhibits the oncogenic JAKs–STAT3 pathway.

These results suggest that the collapse of PLZF expression by the CCL3 derived from fibroblasts accelerates the cell migration and invasion properties of prostate cancer cells. Our results suggest that increasing PLZF could be an attractive strategy for suppressing prostate cancer metastasis as well as for tumor growth.

9.4 FGF AND PCA

In the case of PCa we also have the impact of FGF on the development and enhancement of the malignancy. From Com et al we have the following description:

Aberrant FGF signaling has been implicated in prostate cancer development and progression. With respect to early prostate cancer, multiple studies have shown involvement of dysregulated FGF/FGFR signaling in all developmental stages of prostatic intraepithelial neoplasia (PIN)—carcinoma in situ of the prostate characterized by initial cell proliferation and anaplasia—

followed by invasive carcinoma, angiogenesis, and metastasis. For example, forced expression of constitutively active mutant of FGFR1 leads to development of high-grade PIN lesions.

As we have noted elsewhere[25], HGPIN is not a one way street. It has been seen to be resolvable in cases. Namely, the presence of HGPIN can be eliminated by means and methods yet to be determined. And it appears not to return.

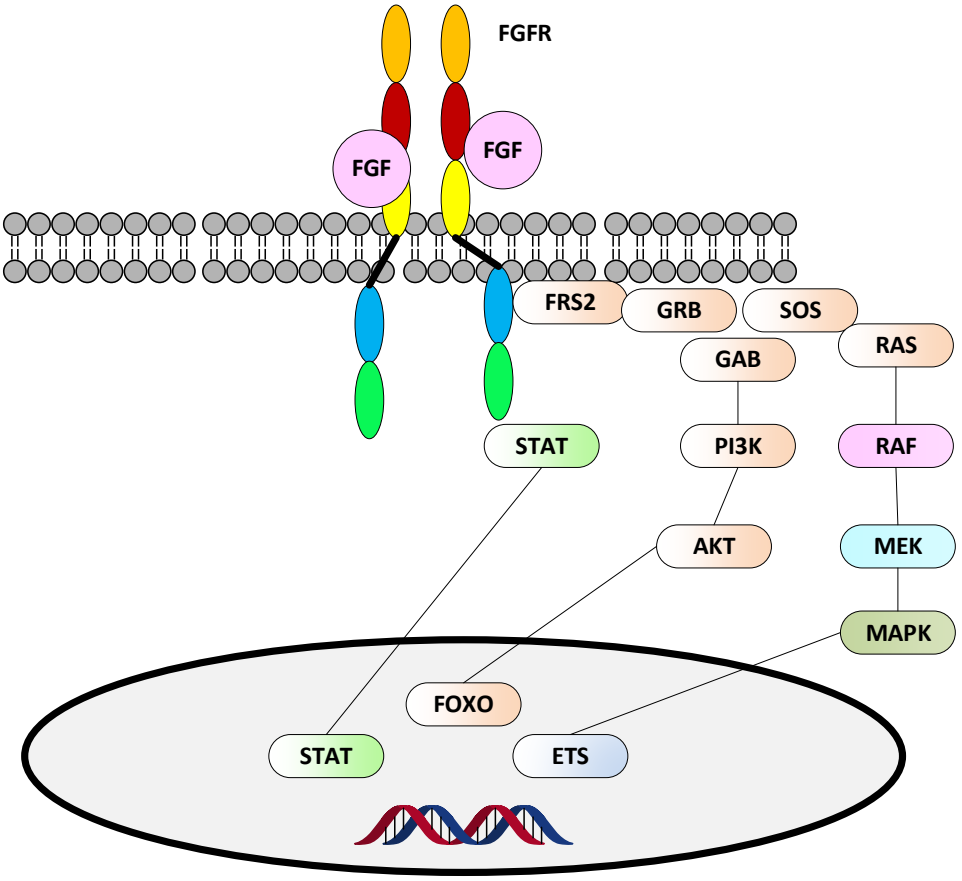
Perturbations of FGF/FGFR pathway signaling also contribute to the development of PIN and prostate cancer through mechanisms that mimic the developmental program. Clear recent evidence that paracrine activation of FGFR in prostate epithelial cells leads to PIN or prostate cancer was provided using a tissue recombination prostate regeneration system. In this model, adult-dissociated normal mouse prostate epithelial cells (mNPE) are combined with embryonic urogenital sinus mesenchyme (UGSM) and grafted under the kidney capsule of a severe combined immunodeficient (SCID) mouse, resulting in the formation of prostate gland–like structures. This model allows for genetic manipulation of the epithelial and mesynchymal compartments independently to study effects of paracrine factors on adult prostate epithelial cells.

In this system, mesenchymal expression of FGF10 leads to FGFR1- dependent PIN or prostate cancer development and enhanced androgen receptor expression in the neoplastic epithelium. Inhibition of epithelial FGFR1 signaling using a dominant-negative FGFR1 led to reversal of the cancer phenotype. In addition, altered expression of FGFR1, but not of FGFR2, has been shown to induce PIN in the juxtaposition of chemical inducers of dimerization (CID) and kinase (JOCK)-1 and -2 transgenic mouse models, respectively. JOCK-1 mice subsequently develop invasive prostate carcinoma and metastasis. Furthermore, conditional deletion of FRS2a in the mouse prostate inhibited the initiation and progression of prostate cancer induced by oncogenic viral proteins. Conditional inactivation of FGFR1 also impairs development of PIN, prostate cancer progression, and metastasis in the TRAMP model.

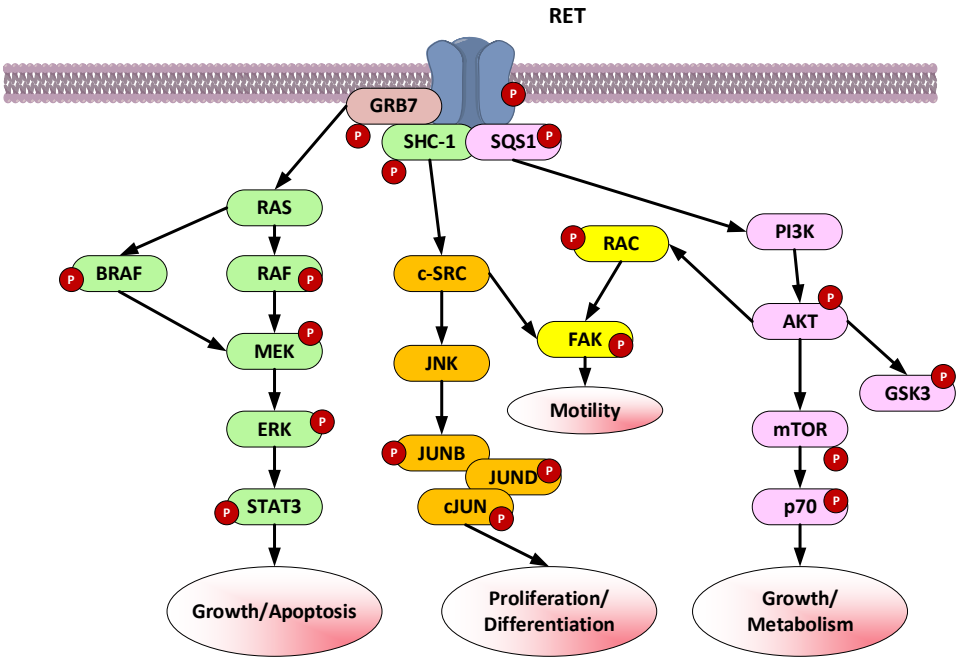
Together, these reports suggest that activation of FGFR1 signaling is sufficient to induce prostate cancer development and progression in mouse models of prostate cancer. It has also been shown that increased production of FGF ligands by prostatic secretory epithelial cells induces autocrine signaling and independence from stromal regulation, stimulating aberrant epithelial growth, and cellular dysplasia. For example, FGF8 and its cognate receptors FGFR1IIIc and FGFR2IIIc are overexpressed in human samples of PIN and prostate cancer compared with controls.

We demonstrate some of these signalling paths below.

25 https://www.researchgate.net/publication/325047485_PROSTATIC_INTRAEPITHELIAL_NEOPLASIA_PROGRESSION_REGRESSION_A_MODEL_FOR_PROSTATE_CANCER



A more complete representation is as follows:


RET
GRB7
SHC-1
SQS1
RAS
BRAF
RAF
MEK
ERK
STAT3
Growth/Apoptosis
c-SRC
JNK
JUNB
JUND
cJUN
Proliferation/
Differentiation
RAC
FAK
Motility
PI3K
AKT
GSK3
mTOR
p70
Growth/
Metabolism

10 OBSERVATIONS

We can now make some observations regarding the studies discussed. Clearly CAFs represent one of several legs upon which cancer cells rely upon for their resistance to attack as well as their proliferation and metastatic potential. The prime message in this Note is that the TME is as critical to understanding cancers as is any of the pathway dynamics withing any cancer cell. Moreover, the understanding of the elements of the TME is essential when considering immunotherapeutics. It is essential to break through the protective barrier that the TME in conjunction with the cancer cells create.

10.1 "WOUND" HEALING

Metaphorically cancer is "the wound which will not heal". As such and in any other wound, the body has developed a means to isolate and combat the wound. However, cancer cells manage to overtake the isolation property and use it for its own proliferation.

From Greaves et al:

Cutaneous wound healing ultimately functions to facilitate barrier restoration following injury-induced loss of skin integrity. It is an evolutionarily conserved, multi-cellular, multi-molecular process involving coordinated interplay between complex signalling networks. Cellular proliferation is recognised as the third stage of this sequence. Within this phase, fibroplasia and angiogenesis are co-dependent processes which must be successfully completed in order to form an evolving extracellular matrix and granulation tissue.

The resultant structures guide cellular infiltration, differentiation and secretory profile within the wound environment and consequently have major influence on the success or failure of wound healing. This review integrates in vitro, animal and human in vivo studies, to provide up to date descriptions of molecular and cellular interactions involved in fibroplasia and angiogenesis. Significant molecular networks include adhesion molecules, proteinases, cytokines and chemokines as well as a plethora of growth factors. These signals are produced by, and affect behaviour of, cells including fibroblasts, fibrocytes, keratinocytes, endothelial cells and inflammatory cells resulting in significant cellular phenotypic and functional plasticity, as well as controlling composition and remodelling of structural proteins including collagen and fibronectin.

The interdependent relationship between angiogenesis and fibroplasia relies on dynamic reciprocity between cellular components, matrix proteins and bioactive molecules. Unbalanced regulation of any one component can have significant consequences resulting in delayed healing, chronic wounds or abnormal scar formation. Greater understanding of angiogenic and fibroplastic mechanisms underlying chronic wound pathogenesis has identified novel therapeutic targets and enabled development of improved treatment strategies including topical growth factors and skin substitutes.

10.2 Integrated Therapeutics

In attempting to defeat the cancer cells one must perforce of the protective nature of the TME also fight the alterations in the TME elements. This therapeutics must be a "cocktail" of therapeutic elements that fight not only the cancer cell via pathway inhibition of immunotherapy methods but also attack the protective TME.

As Lamprecht et al have noted:

The reductionist viewpoint of neoplasia based on the autonomous behavior of tumor cells has been superseded by the mounting evidence that cancer is a complex micro-ecosystem comprising not only the transformed cells but also heterotypic non-cancer cell populations resident in the tumor microenvironment (TME).

This view is providing new opportunities for therapeutic approaches. Previously, stromal fibroblasts enclosing a growing tumor were not at the center of attention as key participants in the process of carcinogenesis. A vast number of studies have discounted this view and showed that the naïve fibroblasts are educated by the adjacent tumor cells to foster their growth program. The "activated" fibroblasts, referred to as myofibroblasts and in the cancer context "cancer-associated fibroblasts" (CAFs), are the most abundant cell population resident in the TME producing an array of oncogenic cytokines and chemokines.

Several cell types have been qualified as progenitors that acquire the CAF phenotype by trans-differentiation explaining, at least in part, the heterogeneity of CAFs in the TME. Apart from normal resident fibroblasts, the list includes pericytes, adipocytes, smooth muscle cells, hepatic and pancreatic stellate cells to name but a few.

Epithelial cancer cells are also able to acquire a CAF-like mesenchymal phenotype via the epithelial–mesenchymal transition (EMT), a trans-differentiation program that strongly favors migration and invasion of cancer cells. Irrespective of their disparate cellular origin and lineage, the ultimate purpose of these progenitoronce morphed into CAFs to sustain the demanding lifestyle of the cancer cell. A note of caution is warranted at this point.

Tumor stroma does not invariably act as a partner in crime but may restrain cancer growth, an inhibitory role shown in murine pancreatic cancer. In this context, the secretion by CAFs of pro-collagen fibrils into the extracellular matrix (ECM)—referred to as desmoplasia—serves as a barrier impeding the growth of cancer cells instead of enhancing their biological aggressiveness.

10.3 Histology and TME

Histologically one examines cancers by looking at the principle cells. At best there may be a side statement regarding the TME and often total disregard for the ECM. The focus of the pathologist is on the aberrant organ cell. We have argued herein that the totality of the TME should and

ultimately must be considered. This is especially true if we seek to employ immunotherapy which can be blocked by the TME elements.

All too often the path report may at best make a passing reference to the TME. Almost all grading systems are independent of any TME environments and furthermore, the path studies almost never address TME markers for cell differentiation.

We would argue that a broader collection of information will be essential for any form of personalized/individualized treatments.

11 APPENDIX: KEY GENES

The following are some key genes as referenced herein and as detailed in NCBI.

Gene	*Function*
AKT	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.
CCL2	This gene is one of several cytokine genes clustered on the q-arm of chromosome 17. Chemokines are a superfamily of secreted proteins involved in immunoregulatory and inflammatory processes. The superfamily is divided into four subfamilies based on the arrangement of N-terminal cysteine residues of the mature peptide. This chemokine is a member of the CC subfamily which is characterized by two adjacent cysteine residues. This cytokine displays chemotactic activity for monocytes and basophils but not for neutrophils or eosinophils. It has been implicated in the pathogenesis of diseases characterized by monocytic infiltrates, like psoriasis, rheumatoid arthritis and atherosclerosis. It binds to chemokine receptors CCR2 and CCR4.
CCL5	This gene is one of several chemokine genes clustered on the q-arm of chromosome 17. Chemokines form a superfamily of secreted proteins involved in immunoregulatory and inflammatory processes. The superfamily is divided into four subfamilies based on the arrangement of the N-terminal cysteine residues of the mature peptide. This chemokine, a member of the CC subfamily, functions as a chemoattractant for blood monocytes, memory T helper cells and eosinophils. It causes the release of histamine from basophils and activates eosinophils. This cytokine is one of the major HIV-suppressive factors produced by CD8+ cells. It functions as one of the natural ligands for the chemokine receptor chemokine (C-C motif) receptor 5 (CCR5), and it suppresses in vitro replication of the R5 strains of HIV-1, which use CCR5 as a coreceptor. Alternative splicing results in multiple transcript variants that encode different isoforms
CD31	The protein encoded by this gene is found on the surface of platelets, monocytes, neutrophils, and some types of T-cells, and makes up a large

Gene	*Function*
	portion of endothelial cell intercellular junctions. The encoded protein is a member of the immunoglobulin superfamily and is likely involved in leukocyte migration, angiogenesis, and integrin activation
CD45	The protein encoded by this gene is a member of the protein tyrosine phosphatase (PTP) family. PTPs are known to be signaling molecules that regulate a variety of cellular processes including cell growth, differentiation, mitosis, and oncogenic transformation. This PTP contains an extracellular domain, a single transmembrane segment and two tandem intracytoplasmic catalytic domains, and Thus, is classified as a receptor type PTP. This PTP has been shown to be an essential regulator of T- and B-cell antigen receptor signaling. It functions through either direct interaction with components of the antigen receptor complexes, or by activating various Src family kinases required for the antigen receptor signaling. This PTP also suppresses JAK kinases, and Thus, functions as a regulator of cytokine receptor signaling. Alternatively spliced transcripts variants of this gene, which encode distinct isoforms, have been reported.
CgA	
CSF-1	The protein encoded by this gene is a cytokine that controls the production, differentiation, and function of macrophages. The active form of the protein is found extracellularly as a disulfide-linked homodimer, and is thought to be produced by proteolytic cleavage of membrane-bound precursors. The encoded protein may be involved in development of the placenta. Alternate splicing results in multiple transcript variants
CXCL12	This antimicrobial gene encodes a stromal cell-derived alpha chemokine member of the intercrine family. The encoded protein functions as the ligand for the G-protein coupled receptor, chemokine (C-X-C motif) receptor 4, and plays a role in many diverse cellular functions, including embryogenesis, immune surveillance, inflammation response, tissue homeostasis, and tumor growth and metastasis. Mutations in this gene are associated with resistance to human immunodeficiency virus type 1 infections. Multiple transcript variants encoding different isoforms have been found for this gene.
EGF	This gene encodes a member of the epidermal growth factor superfamily. The encoded preproprotein is proteolytically processed 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 with high affinity to the 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. Alternative splicing results in multiple transcript variants, at least one of which encodes a preproprotein that is proteolytically processed
EGFR	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

Gene	*Function*
	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.
FGF1	The protein encoded by this gene is a member of the fibroblast growth factor (FGF) family. FGF family members possess broad mitogenic and cell survival activities, and are involved in a variety of biological processes, including embryonic development, cell growth, morphogenesis, tissue repair, tumor growth and invasion. This protein functions as a modifier of endothelial cell migration and proliferation, as well as an angiogenic factor. It acts as a mitogen for a variety of mesoderm- and neuroectoderm-derived cells in vitro, Thus, is thought to be involved in organogenesis. Multiple alternatively spliced variants encoding different isoforms have been described.
FGF2	The protein encoded by this gene is a member of the fibroblast growth factor (FGF) family. FGF family members bind heparin and possess broad mitogenic and angiogenic activities. This protein has been implicated in diverse biological processes, such as limb and nervous system development, wound healing, and tumor growth. The mRNA for this gene contains multiple polyadenylation sites, and is alternatively translated from non-AUG (CUG) and AUG initiation codons, resulting in five different isoforms with distinct properties. The CUG-initiated isoforms are localized in the nucleus and are responsible for the intracrine effect, whereas, the AUG-initiated form is mostly cytosolic and is responsible for the paracrine and autocrine effects of this FGF.
FGF3	The protein encoded by this gene is a member of the fibroblast growth factor (FGF) family. FGF family members possess broad mitogenic and cell survival activities and are involved in a variety of biological processes including embryonic development, cell growth, morphogenesis, tissue repair, tumor growth and invasion. This gene was identified by its similarity with mouse fgf3/int-2, a proto-oncogene activated in virally induced mammary tumors in the mouse. Frequent amplification of this gene has been found in human tumors, which may be important for neoplastic transformation and tumor progression. Studies of the similar genes in mouse and chicken suggested the role in inner ear formation.
FGF7	The protein encoded by this gene is a member of the fibroblast growth factor (FGF) family. FGF family members possess broad mitogenic and cell survival activities, and are involved in a variety of biological processes, including embryonic development, cell growth, morphogenesis, tissue repair, tumor growth and invasion. This protein is a potent epithelial cell-specific growth factor, whose mitogenic activity is predominantly exhibited in keratinocytes but not in fibroblasts and endothelial cells. Studies of mouse and rat homologs of this gene

Gene	*Function*
	implicated roles in morphogenesis of epithelium, reepithelialization of wounds, hair development and early lung organogenesis.
FGFR1	The protein encoded by this gene is a member of the fibroblast growth factor receptor (FGFR) family, where amino acid sequence is highly conserved between members and throughout evolution. FGFR family members differ from one another in their ligand affinities and tissue distribution. A full-length representative protein consists 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 both acidic and basic fibroblast growth factors and is involved in limb induction. Mutations in this gene have been associated with Pfeiffer syndrome, Jackson-Weiss syndrome, Antley-Bixler syndrome, osteoglophonic dysplasia, and autosomal dominant Kallmann syndrome 2. Chromosomal aberrations involving this gene are associated with stem cell myeloproliferative disorder and stem cell leukemia lymphoma syndrome. Alternatively spliced variants which encode different protein isoforms have been described; However, not all variants have been fully characterized.
FGFR2	The protein encoded by this gene is a member of the fibroblast growth factor receptor family, where amino acid sequence is highly conserved between members and throughout evolution. FGFR family members differ from one another in their ligand affinities and tissue distribution. A full-length representative protein consists 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 is a high-affinity receptor for acidic, basic and/or keratinocyte growth factor, depending on the isoform. Mutations in this gene are associated with Crouzon syndrome, Pfeiffer syndrome, Craniosynostosis, Apert syndrome, Jackson-Weiss syndrome, Beare-Stevenson cutis gyrata syndrome, Saethre-Chotzen syndrome, and syndromic craniosynostosis. Multiple alternatively spliced transcript variants encoding different isoforms have been noted for this gene.
FGFR3	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.

Gene	*Function*
	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. Mutations in this gene lead to craniosynostosis and multiple types of skeletal dysplasia.
FGFR4	The protein encoded by this gene is a tyrosine kinase and cell surface receptor for fibroblast growth factors. The encoded protein is involved in the regulation of several pathways, including cell proliferation, cell differentiation, cell migration, lipid metabolism, bile acid biosynthesis, vitamin D metabolism, glucose uptake, and phosphate homeostasis. This protein consists 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 interacts with fibroblast growth factors, setting in motion a cascade of downstream signals, ultimately influencing mitogenesis and differentiation
GR1	
HGF	This gene encodes a protein that binds to the hepatocyte growth factor receptor to regulate cell growth, cell motility and morphogenesis in numerous cell and tissue types. Alternative splicing results in multiple transcript variants, at least one of which encodes a preproprotein that is proteolytically processed to generate alpha and beta chains, which form the mature heterodimer. This protein is secreted by mesenchymal cells and acts as a multi-functional cytokine on cells of mainly epithelial origin. This protein also plays a role in angiogenesis, tumorogenesis, and tissue regeneration. Although the encoded protein is a member of the peptidase S1 family of serine proteases, it lacks peptidase activity. Mutations in this gene are associated with nonsyndromic hearing loss
HIF	
IDO	This gene encodes indoleamine 2,3-dioxygenase (IDO) - a heme enzyme that catalyzes the first and rate-limiting step in tryptophan catabolism to N-formyl-kynurenine. This enzyme acts on multiple tryptophan substrates including D-tryptophan, L-tryptophan, 5-hydroxy-tryptophan, tryptamine, and serotonin. This enzyme is thought to play a role in a variety of pathophysiological processes such as antimicrobial and antitumor defense, neuropathology, immunoregulation, and antioxidant activity. Through its expression in dendritic cells, monocytes, and macrophages this enzyme modulates T-cell behavior by its peri-cellular catabolization of the essential amino acid tryptophan
IL-18	
IL-1β	The protein encoded by this gene is a member of the interleukin 1 cytokine family. This cytokine is produced by activated macrophages as a proprotein, which is proteolytically processed to its active form by caspase 1 (CASP1/ICE). This cytokine is an important mediator of the

Gene	*Function*
	inflammatory response, and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis. The induction of cyclooxygenase-2 (PTGS2/COX2) by this cytokine in the central nervous system (CNS) is found to contribute to inflammatory pain hypersensitivity. This gene and eight other interleukin 1 family genes form a cytokine gene cluster on chromosome 2
IL-6	This gene encodes a cytokine that functions in inflammation and the maturation of B cells. In addition, the encoded protein has been shown to be an endogenous pyrogen capable of inducing fever in people with autoimmune diseases or infections. The protein is primarily produced at sites of acute and chronic inflammation, where it is secreted into the serum and induces a transcriptional inflammatory response through interleukin 6 receptor, alpha. The functioning of this gene is implicated in a wide variety of inflammation-associated disease states, including suspectibility to diabetes mellitus and systemic juvenile rheumatoid arthritis. Alternative splicing results in multiple transcript variants. [provided by RefSeq, Dec 2015] Expression Broad expression in urinary bladder
IL-8	
MMP-9	Proteins of the matrix metalloproteinase (MMP) family are involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling, as well as in disease processes, such as arthritis and metastasis. Most MMP's are secreted as inactive proproteins which are activated when cleaved by extracellular proteinases. The enzyme encoded by this gene degrades type IV and V collagens. Studies in rhesus monkeys suggest that the enzyme is involved in IL-8-induced mobilization of hematopoietic progenitor cells from bone marrow, and murine studies suggest a role in tumor-associated tissue remodeling
PDGF	This gene encodes a member of the protein family comprised of both platelet-derived growth factors (PDGF) and vascular endothelial growth factors (VEGF). The encoded preproprotein is proteolytically processed to generate platelet-derived growth factor subunit A, which can homodimerize, or alternatively, heterodimerize with the related platelet-derived growth factor subunit B. These proteins bind and activate PDGF receptor tyrosine kinases, which play a role in a wide range of developmental processes. Alternative splicing results in multiple transcript variants.
PGE2	
SDF1 (CXCL12)	This antimicrobial gene encodes a stromal cell-derived alpha chemokine member of the intercrine family. The encoded protein functions as the ligand for the G-protein coupled receptor, chemokine (C-X-C motif) receptor 4, and plays a role in many diverse cellular functions, including embryogenesis, immune surveillance, inflammation response, tissue

Gene	*Function*
	homeostasis, and tumor growth and metastasis. Mutations in this gene are associated with resistance to human immunodeficiency virus type 1 infections. Multiple transcript variants encoding different isoforms have been found for this gene
SOX2	This intronless gene encodes a member of the SRY-related HMG-box (SOX) family of transcription factors involved in the regulation of embryonic development and in the determination of cell fate. The product of this gene is required for stem-cell maintenance in the central nervous system, and also regulates gene expression in the stomach. Mutations in this gene have been associated with optic nerve hypoplasia and with syndromic microphthalmia, a severe form of structural eye malformation. This gene lies within an intron of another gene called SOX2 overlapping transcript (SOX2OT)
TGF-β	This gene encodes a secreted ligand of the TGF-beta (transforming growth factor-beta) superfamily of proteins. Ligands of this family bind various TGF-beta receptors leading to recruitment and activation of SMAD family transcription factors that regulate gene expression. The encoded preproprotein is proteolytically processed to generate a latency-associated peptide (LAP) and a mature peptide, and is found in either a latent form composed of a mature peptide homodimer, a LAP homodimer, and a latent TGF-beta binding protein, or in an active form consisting solely of the mature peptide homodimer. The mature peptide may also form heterodimers with other TGFB family members. This encoded protein regulates cell proliferation, differentiation and growth, and can modulate expression and activation of other growth factors including interferon gamma and tumor necrosis factor alpha. This gene is frequently upregulated in tumor cells, and mutations in this gene result in Camurati-Engelmann disease
TNF	This gene encodes a multifunctional proinflammatory cytokine that belongs to the tumor necrosis factor (TNF) superfamily. This cytokine is mainly secreted by macrophages. It can bind to, and Thus, functions through its receptors TNFRSF1A/TNFR1 and TNFRSF1B/TNFBR. This cytokine is involved in the regulation of a wide spectrum of biological processes including cell proliferation, differentiation, apoptosis, lipid metabolism, and coagulation. This cytokine has been implicated in a variety of diseases, including autoimmune diseases, insulin resistance, and cancer. Knockout studies in mice also suggested the neuroprotective function of this cytokine.
TSLP	This gene encodes a hemopoietic cytokine proposed to signal through a heterodimeric receptor complex composed of the thymic stromal lymphopoietin receptor and the IL-7R alpha chain. It mainly impacts myeloid cells and induces the release of T cell-attracting chemokines from monocytes and enhances the maturation of CD11c(+) dendritic cells. The protein promotes T helper type 2 (TH2) cell responses that are associated with immunity in various inflammatory diseases, including asthma,

Gene	*Function*
	allergic inflammation and chronic obstructive pulmonary disease. The protein is therefore considered a potential therapeutic target for the treatment of such diseases. Alternative splicing of this gene results in multiple transcript variants
TWIST	This gene encodes a basic helix-loop-helix (bHLH) transcription factor that plays an important role in embryonic development. The encoded protein forms both homodimers and heterodimers that bind to DNA E box sequences and regulate the transcription of genes involved in cranial suture closure during skull development. This protein may also regulate neural tube closure, limb development and brown fat metabolism. This gene is hypermethylated and overexpressed in multiple human cancers, and the encoded protein promotes tumor cell invasion and metastasis. Mutations in this gene cause Saethre-Chotzen syndrome in human patients, which is characterized by craniosynostosis, ptosis and hypertelorism
VEGF	This gene is a member of the PDGF/VEGF growth factor family. It encodes a heparin-binding protein, which exists as a disulfide-linked homodimer. This growth factor induces proliferation and migration of vascular endothelial cells, and is essential for both physiological and pathological angiogenesis. Disruption of this gene in mice resulted in abnormal embryonic blood vessel formation. This gene is upregulated in many known tumors and its expression is correlated with tumor stage and progression. Elevated levels of this protein are found in patients with POEMS syndrome, also known as Crow-Fukase syndrome. Allelic variants of this gene have been associated with microvascular complications of diabetes 1 (MVCD1) and atherosclerosis. Alternatively spliced transcript variants encoding different isoforms have been described. There is also evidence for alternative translation initiation from upstream non-AUG (CUG) codons resulting in additional isoforms. A recent study showed that a C-terminally extended isoform is produced by use of an alternative in-frame translation termination codon via a stop codon readthrough mechanism, and that this isoform is antiangiogenic. Expression of some isoforms derived from the AUG start codon is regulated by a small upstream open reading frame, which is located within an internal ribosome entry site

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