EMT and Cancers

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White Paper No 157
January, 2019

The epithelial to mesenchymal transition, EMT, is a process that has been closely linked to cancer metastasis. We examine its current understanding and make some observations. It close relationship to immune drivers may make it an interesting target for immunotherapy.

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1 INTRODUCTION

When I was young and my father had returned from the Navy in WWII he had a phrase he used frequently to admonish my at times less than nest tendencies. Namely: "a place for everything and everything in its place". I thought I knew what he was saying but it was not until I started to understand cancer metastasis that this truly rang a bell. Cancer is not "neat". It just drops stuff all over the place, sending cells hither and thither, never putting things back where they belong.

We examine another process which is linked to cancer and metastasis, namely the Epithelial to Mesenchymal Transition, EMT. As we have noted in many other areas we have examined this has been argued to have significant therapeutic interest. There has been some examination here as of later but there is limited clinical application. What this area does do is shine a light on the issue of cell location and lost of location stability as an integral part of cell carcinogenesis.

Cells express genes in different ways depending when and where they are. Epithelial cells generally express genes that allow the cell to perform a specific function and to do so at a specific location. However from time to time, such as in the growth phase of an organism, this stable phenotype is suppressed and the cell has a characteristic that allows it to move freely as a mesenchymal cell. Thus transitions from mesenchymal to epithelial phenotypes are stabilizing transitions in a maturing organism (called MET). The reverse, EMT, are generally destabilizing transitions. For example a melanocyte with E cadherin expressed binds to the other keratinocytes and remain stable in the skin. When E cadherin is not expressed but N cadherin is, the melanocytes bind together and then wander, often first upward creating a carcinoma in situ, then downward creating a melanoma. Thus as expression of genes is effected the process of EMT allows for movement and thus metastasis.

In this note, we examine some of the recent advances understanding this process, especially as applied to several somatic malignancies. There is also the consideration of using EMT mechanisms as a means to target therapeutics to mitigate metastasis. The state of the art is still somewhat early but it does provide an interesting alternative. This paper is not meant to be comprehensive but suggestive.

Weinberg presents a detailed description of the EMT as a part of metastasis[1].

As Heerboth et al note:

EMT and MET comprise the processes by which cells transit between epithelial and mesenchymal states, and they play integral roles in both normal development and cancer metastasis. This article reviews these processes and the molecular pathways that contribute to them.

First, we compare embryogenesis and development with cancer metastasis.

[1] Weinberg pp 657-669

We then discuss the signaling pathways and the differential expression and down-regulation of receptors in both tumor cells and stromal cells, which play a role in EMT and metastasis.

We further delve into the clinical implications of EMT and MET in several types of tumors, and lastly, we discuss the role of epigenetic events that regulate EMT/MET processes.

We hypothesize that reversible epigenetic events regulate both EMT and MET, and thus, also regulate the development of different types of metastatic cancers.

The above is more of an outline of the issues that can be considered. Namely: (i) benign EMT processes versus malignant, (ii) downregulation and control of EMT pathways, (iii) specific EMT effects in specific cancers, and (iv) reversible controls regulating metastasis.

As Radisky notes:

The epithelial-mesenchymal transition (EMT) is an orchestrated series of events in which cell-cell and cell-extracellular matrix (ECM) interactions are altered to release epithelial cells from the surrounding tissue, the cytoskeleton is reorganized to confer the ability to move through a three-dimensional ECM, and a new transcriptional program is induced to maintain the mesenchymal phenotype.

Essential for embryonic development, EMT is nevertheless potentially destructive if deregulated, and it is becoming increasingly clear that inappropriate utilization of EMT mechanisms is an integral component of the progression of many tumors of epithelial tissues. Structural integrity is a key property of epithelial tissues: external epithelia serve as protective barriers against environmental hazards, and internal epithelia create defined and physiologically controlled subdomains within the organism. Epithelial structure is maintained by cell-cell interactions.

These involve tight junctions, cadherin based adherens junctions that are connected to the actin cytoskeleton, gap junctions that allow direct chemical interactions between neighboring cells, and desmosomes connected to the intermediate filament cytoskeleton, and cell-ECM interactions mediated by integrins and other molecules.

In this paper we address the following:

1. What is EMT and how does it function?
2. What are the critical drivers of the EMT process?
3. How does EMT effect a cancerous process?
4. What are the pathway elements involved in EMT?
5. What are the specifics of various cancers and EMT?
6. What is the interaction between the immune system and the EMT process?
7. What role does chronic inflammation play in EMT activation and in turn cancer?

8. What are the therapeutic opportunities available in the EMT context?

9. Does the presence of blood borne EMT markers present a diagnostic, prognostic, and therapeutic opportunity?

2 EMT

EMT is a process whereby a cell changes from a stable cell in a well defined matrix to a cell which has the ability to move about in a relatively unstructured manner. In essence the EMT process enables a metastatic change. We summarize some of these features herein.

2.1 BASIC CONCEPTS

EMT is simply the process whereby cells lose the ability to be at the right place at the right time. From Kalluri and Weinberg we have a definition:

An epithelial-mesenchymal transition (EMT) is a biologic process that allows a polarized epithelial cell, which normally interacts with basement membrane via its basal surface, to undergo multiple biochemical changes that enable it to assume a mesenchymal cell phenotype, which includes enhanced migratory capacity, invasiveness, elevated resistance to apoptosis, and greatly increased production of ECM (extra cellular matrix) components.

The completion of an EMT is signaled by the degradation of underlying basement membrane and the formation of a mesenchymal cell that can migrate away from the epithelial layer in which it originated.

Thus many cells are organized in a certain manner to effect certain functions. In the prostate, a glandular organ, there are basal and luminal cells surrounding the glands wherein secretion occurs. In the case of high grade PIN for example, the cells start to proliferate and no longer align properly. Then they slowly depart and create for wont of a better term, move out. They continue:

A number of distinct molecular processes are engaged in order to initiate an EMT and enable it to reach completion. These include activation of transcription factors, expression of specific cell-surface proteins, reorganization and expression of cytoskeletal proteins, production of ECM-degrading enzymes, and changes in the expression of specific microRNAs. In many cases, the involved factors are also used as biomarkers to demonstrate the passage of a cell through an EMT..

The pioneering work of Elizabeth Hay first described an "epithelial mesenchymal transformation" using a model of chick primitive streak formation. In the intervening time, the term "transformation" has been replaced with "transition," reflecting in part the reversibility of the process and the fact that it is distinct from neoplastic transformation.

The phenotypic plasticity afforded by an EMT is revealed by the occurrence of the reverse process — a mesenchymal- epithelial transition (MET), which involves the conversion of mesenchymal cells to epithelial derivatives. Relatively little is known about this process; the best-studied example is the MET associated with kidney formation, which is driven by genes such as paired box 2 (Pax2), bone morphogenetic protein 7 (Bmp7), and Wilms tumor 1 (Wt1).

From Kalluri and Weinberg we have three types of MET cells are discussed:

(A) Type 1 EMT is associated with implantation and embryonic gastrulation and gives rise to the mesoderm and endoderm and to mobile neural crest cells. The primitive epithelium, specifically the epiblast, gives rise to primary mesenchyme via an EMT. This primary mesenchyme can be re-induced to form secondary epithelia by a MET. It is speculated that such secondary epithelia may further differentiate to form other types of epithelial tissues and undergo subsequent EMT to generate the cells of connective tissue, including astrocytes, adipocytes, chondrocytes, osteoblasts, and muscle cells.

(B) EMTs are re-engaged in the context of inflammation and fibrosis and represent the type 2 EMTs. Unlike the type 1 EMT, the type 2 EMT is expressed over extended periods of time and can eventually destroy an affected organ if the primary inflammatory insult is not removed or attenuated.

(C) Finally, the secondary epithelia associated with many organs can transform into cancer cells that later undergo the EMTs that enable invasion and metastasis, thereby representing type 3 EMTs.

Namely this details the three types; (i) those involved in a developing organism, (ii) those involved in a repairing organism, and (iii) those involved in a metastasizing organism. There is a similarity amongst these three.

As Kong et al have noted:

Cancer stem cells (CSCs) are cells within a tumor that possess the capacity to self-renew and *maintain tumor-initiating capacity through differentiation into the heterogeneous lineages of cancer cells that comprise the whole tumor. These tumor-initiating cells could provide a resource for cells that cause tumor recurrence after therapy. Although the cell origin of CSCs remains to be fully elucidated, mounting evidence has demonstrated that Epithelial-to-Mesenchymal Transition (EMT), induced by different factors, is associated with tumor aggressiveness and metastasis and these cells share molecular characteristics with CSCs, and thus are often called cancer stem-like cells or tumor-initiating cells.*

The acquisition of an EMT phenotype is a critical process for switching early stage carcinomas into invasive malignancies, which is often associated with the loss of epithelial differentiation and gain of mesenchymal phenotype. Recent studies have demonstrated that EMT plays a critical role not only in tumor metastasis but also in tumor recurrence and that it is tightly linked with the biology of cancer stem-like cells or cancer-initiating cells. Here we will succinctly summarize the state-of-our-knowledge regarding the molecular similarities between cancer stem-like cells or CSCs and EMT-phenotypic cells that are associated with tumor aggressiveness focusing on solid tumors.

2.2 EMT TRANSCRIPTION FACTORS

Transcription factors, TF, are proteins which regulate the conversion of DNA to mRNA. They have the capacity to turn the expression on or off and regulate the speed of such conversion. TF play a role in EMT processes. The details of how these TF function are not considered here. In fact, their specific operations are complex and as of yet not fully understood. We thus just consider them functionally as elements in an overall system.

As Mladinich et al note:

Epithelial-to-mesenchymal transition (EMT) was originally discovered for its role during gastrulation of embryogenesis, but more recently EMT activation has been detected in abnormal somatic cells such as cancer cells.

In healthy subjects, differentiated epithelial cells form tight cell to cell adhesions with neighboring cells, as well as contacts with the basement membrane to compose the epithelium. This continuous layer of cells creates a border that separates the environment's apical and basal surface to the epithelium.

This border is dissolved when cells undergo EMT, a process that involves the transcriptional repression of epithelial markers, such as E-cadherin, and expression of mesenchymal markers such as N-cadherin, vimentin, and fibronectin. The resultant mesenchymal cells lose cell-to-cell adhesion and cell polarity and gain migratory and invasive capabilities. Positive correlations between EMT-associated genes and poor disease outcomes have been reported in various human cancer types….

Here the note is that TF are involved in suppressing certain factors which result in loss of patency of the cell.

EMT activation can be induced by genetic mutations occurring in cancer cells or external environmental stimuli. In both cases, several signaling pathways including transforming growth factor beta (TGF-β), Notch, Wnt, and integrin are known to activate EMT through transcriptional repression of E-cadherin. E-cadherin functions as a key gatekeeper of the epithelial state. Loss or downregulation of E-cadherin has been considered to be a hallmark of EMT.

E-cadherin is mutated or downregulated in various human tumors. Apart from the genetic mutation, downregulation of E-cadherin can be mediated by epigenetic silencing as well as EMT-controlling TFs including Snail (Snail1), Slug (Snail2), Twist, zinc finger E-box-binding (Zeb)1/2, and others. The Snail and Twist protein families are the most intensively studied EMT-TFs and have been functionally linked to CSC activation.

The above three TF are thus a focus of attention. Perhaps they may be useful targets for a therapeutic approach. We discuss them in some length below.

2.3 SOME SPECIFIC TF

We now consider several specific TF related to EMT functioning.

2.3.1 SLUG

We begin with SLUG.

SLUG[2] (SNAI2, WS2D; SLUGH; SLUGH1; SNAIL2): This gene encodes a member of the Snail family of C2H2-type zinc finger transcription factors. The encoded protein acts as a transcriptional repressor that binds to E-box motifs and is also likely to repress E-cadherin transcription in breast carcinoma. This protein is involved in epithelial-mesenchymal transitions and has antiapoptotic activity. Mutations in this gene may be associated with sporadic cases of neural tube defects.

From Zhao et al:

Snail family transcriptional repressor 2 (SNAI2), also known as SLUG, belongs to the highly conserved Snail/Scratch superfamily, which includes SNAI1 (SNAIL), SNAI3 (SMUC), and SCRTs etc. Mammalian SNAI2 has C2H2 type zinc fingers in its carboxyl-terminal region and highly conserved SNAG (Snail/Gfi) domain in the amino-terminal region. SNAI2 binds to the E-box-containing promoter of its downstream target genes through its C-terminal, and acts as a transcriptional repressor depending on the N-terminal SNAG domain that interacts with co-repressors. E-cadherin is one of the well-known target genes negatively regulated by SNAI2. Since E-cadherin is indispensable in the maintenance of epithelial status, SNAI2 is regarded as inducers in epithelial to mesenchymal transition (EMT) in embryogenesis and tumorigenesis.

2.3.2 SNAIL

The second we consider is SNAIL. He we discuss its functions:

SNAIL[3] (SNAI1, SNA; SNAH; SNAIL; SLUGH2; SNAIL1; dJ710H13.1) : The Drosophila embryonic protein snail is a zinc finger transcriptional repressor which downregulates the expression of ectodermal genes within the mesoderm. The nuclear protein encoded by this gene is structurally similar to the Drosophila snail protein, and is also thought to be critical for mesoderm formation in the developing embryo. At least two variants of a similar processed pseudogene have been found on chromosome 2.

As Jagle et al note:

Phenotypic conversion of tumor cells through epithelial-mesenchymal transition (EMT) requires massive gene expression changes. How these are brought about is not clear. Here we examined the impact of the EMT master regulator SNAIL1 on the FOXA family of transcription factors which are distinguished by their particular competence to induce chromatin reorganization for

[2] https://www.ncbi.nlm.nih.gov/gene/6591

[3] https://www.ncbi.nlm.nih.gov/gene/6615

the activation of transcriptional enhancer elements. We show that the expression of SNAIL1 and FOXA genes is anticorrelated in transcriptomes of colorectal tumors and cell lines.

In cellular EMT models, ectopically expressed Snail1 directly represses FOXA1 and triggers downregulation of all FOXA family members, suggesting that loss of FOXA expression promotes EMT.

Indeed, cells with CRISPR/Cas9-induced FOXA-deficiency acquire mesenchymal characteristics. Furthermore, ChIP-seq data analysis of FOXA chromosomal distribution in relation to chromatin structural features which characterize distinct states of transcriptional activity, revealed preferential localization of FOXA factors to transcriptional enhancers at signature genes that distinguish epithelial from mesenchymal colon tumors.

We have discussed FOXA1 functions previously. We review it here again. From Jin et al we have:

FoxA1 (FOXA1), also named HNF-3a, is a winged-helix transcription factor of the forkhead family. It plays essential roles in the epithelial differentiation and development of a number of organs including the pancreas, prostate, and breast (1–6). For example, while FoxA1-knockout mice are developmentally lethal, conditional FoxA1 knockout in the mouse prostate results in severely altered ductal development that contains immature epithelial cells surrounded by abnormally thick stromal layers.

Concordantly, in the adult prostate, FoxA1 has also been tightly linked to the maintenance of the prostate epithelial phenotype and the expression of prostatespecific genes. This is mediated through its regulation of the androgen receptor (AR) transcriptional activities (8, 9). As a pioneering factor, FoxA1 opens up compact chromatin to facilitate subsequent AR recruitment.

Genome-wide location analysis of prostate cancer cells have shown that FoxA1 preoccupies lineage-specific enhancers even before androgen stimulation and cooccupies a majority of AR binding sites in androgen-treated cells. FoxA1 is thus indispensable for defining a prostatic AR program and is critical to prostate development, function, as well as malignant transformation.
From Gerhardt et al we have:

Forkhead box protein A1 (FOXA1) modulates the transactivation of steroid hormone receptors and thus may influence tumor growth and hormone responsiveness in prostate cancer. We therefore investigated the correlation of FOXA1 expression with clinical parameters, prostate-specific antigen (PSA) relapse-free survival, and hormone receptor expression in a large cohort of prostate cancer patients at different disease stages. FOXA1 expression did not differ significantly between benign glands from the peripheral zone and primary peripheral zone prostate carcinomas.

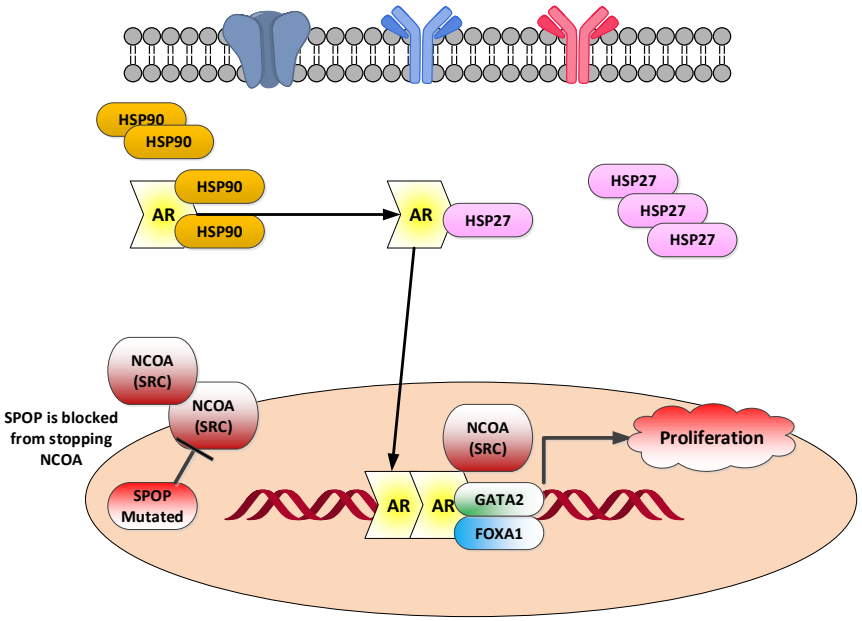
However, FOXA1 was overexpressed in metastases and particularly in castration-resistant cases, but was expressed at lower levels in both normal and neoplastic transitional zone tissues. FOXA1 levels correlated with higher pT stages and Gleason scores, as well as with androgen (AR) and estrogen receptor expression. Moreover, FOXA1 overexpression was associated with

faster biochemical disease progression, which was pronounced in patients with low AR levels. Finally, siRNA-based knockdown of FOXA1 induced decreased cell proliferation and migration.

Moreover, in vitro tumorigenicity was inducible by ARs only in the presence of FOXA1, substantiating a functional cooperation between FOXA1 and AR.

In conclusion, FOXA1 expression is associated with tumor progression, dedifferentiation of prostate cancer cells, and poorer prognosis, as well as with cellular proliferation and migration and with AR signaling. These findings suggest FOXA1 overexpression as a novel mechanism inducing castration resistance in prostate cancer.

From Wyatt and Gleave we have the following descriptive of the pathway blocking impact of SPOP and FOXA1. This is shown below for CRPC.



Now Wyatt and Gleave specifically note regarding FOXA1:

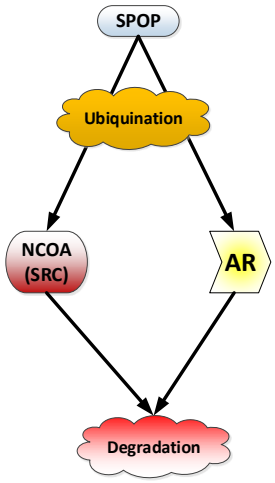
The forkhead protein FOXA1 is a critical interacting partner of the AR, functioning as a pioneer factor to modulate chromatin accessibility and facilitate transcription. In prostate cancer, FOXA1 is capable of specifying unique AR binding sites and has an AR-independent function as a metastasis regulator. Although it can be genomically amplified, deleted, or mutated in CRPC patients, suggesting complex context-dependent, the precedent set by the development of a FOXM1 inhibitor suggests that forkhead protein modulation in prostate cancer might hold promise.

Interestingly, FOXA1 and AR co-localize on chromatin with GATA2, a transcription factor that enhances recruitment of NCOAs to the AR complex. Additionally, at the transcriptional level, there appears to be a complex feedback balance between GATA2 and the AR itself, since GATA2 is repressed by the AR and androgen, but is necessary for optimal expression of the AR. High

GATA2 expression predicts poor outcome in prostate cancer patients and further promotes the concept of therapeutically targeting the AR transcriptional complex in CRPC patients.

A promising contemporary strategy to disrupt AR in this manner is to use bromodomain inhibitors (e.g. JQ1) to inhibit the chromatin reader BRD4 that interacts with the N-terminal domain of the AR. Preclinical studies have shown that JQ1 disrupts AR- mediated gene transcription in CRPC models, significantly reducing tumour volume relative to controls.

They also depict the normal process of ubiquitination and elimination as shown below. This assumes a normal SPOP and FOXA1.



2.3.3 TWIST

TWIST (TWIST, RS; CSO; SCS; ACS3; CRS1; BPES2; BPES3; SWCOS; TWIST; bHLHa38)[4]: 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.

From Mladinich et al:

Twist is a basic helix-loop-helix TF originally shown to be central to embryonic development and later found to be highly expressed in a wide array of metastatic cancers. Further functional analyses establish Twist as a master regulator of cancer metastasis by inducing EMT, increasing tumor cell migration and invasion.

[4] https://www.ncbi.nlm.nih.gov/gene/7291

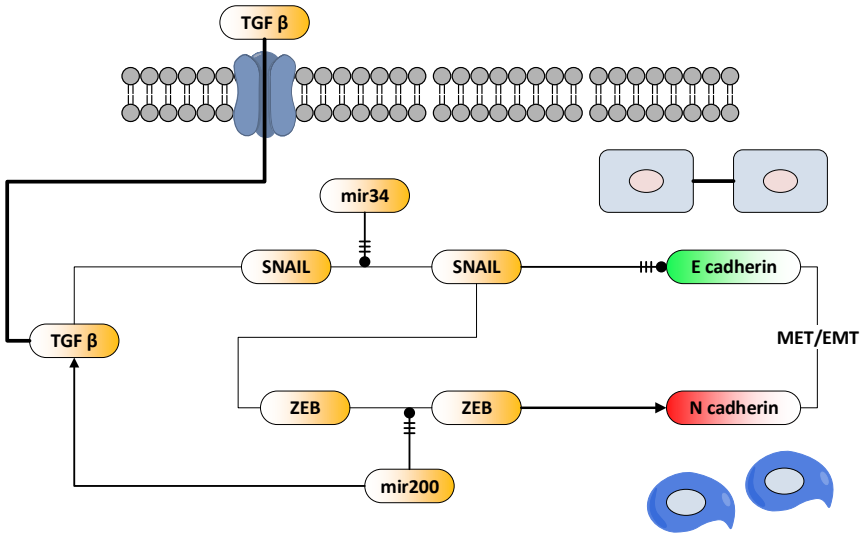
Mechanistically, Twist binds to promoter regions and enhance gene transcription of Slug, subsequently leading to gene repression of E-cadherin Twist can also indirectly repress E-cadherin expression through recruitment of the methyltransferase SET8 that methylates histones for gene silencing.

Apart from its EMT-including ability, Twist can work in concert with BMI1, a polycomb-group repressor complex protein, to orchestrate stem cell self-renewal by direct induction of BMI1 gene expression. In view of Twist's versatile roles in regulating cancer stemness and its influence on other EMT-TFs such as Slug, targeting Twist has been considered as a compelling approach for CSC-based therapy.

As Yang et al note:

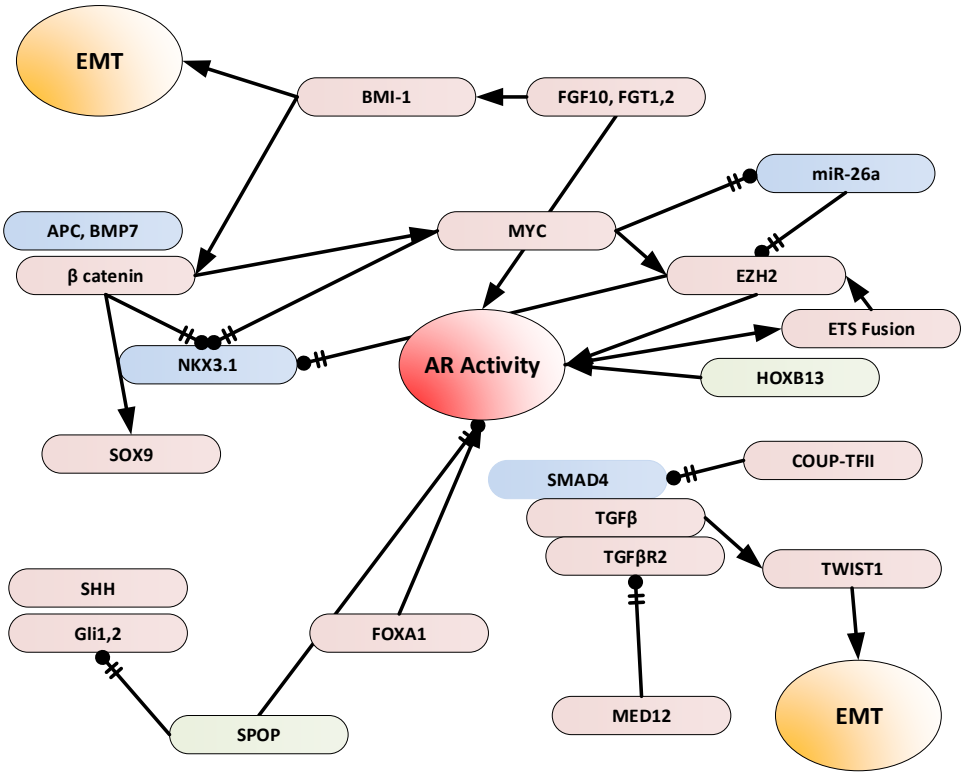
Metastasis is a multistep process during which cancer cells disseminate from the site of primary tumors and establish secondary tumors in distant organs. In a search for key regulators of metastasis in a murine breast tumor model, we have found that the transcription factor Twist, a master regulator of embryonic morphogenesis, plays an essential role in metastasis. Suppression of Twist expression in highly metastatic mammary carcinoma cells specifically inhibits their ability to metastasize from the mammary gland to the lung.

Ectopic expression of Twist results in loss of E-cadherin-mediated cell-cell adhesion, activation of mesenchymal markers, and induction of cell motility, suggesting that Twist contributes to metastasis by promoting an epithelial-mesenchymal transition (EMT). In human breast cancers, high level of Twist expression is correlated with invasive lobular carcinoma, a highly infiltrating tumor type associated with loss of E-cadherin expression. These results establish a mechanistic link between Twist, EMT, and tumor metastasis.

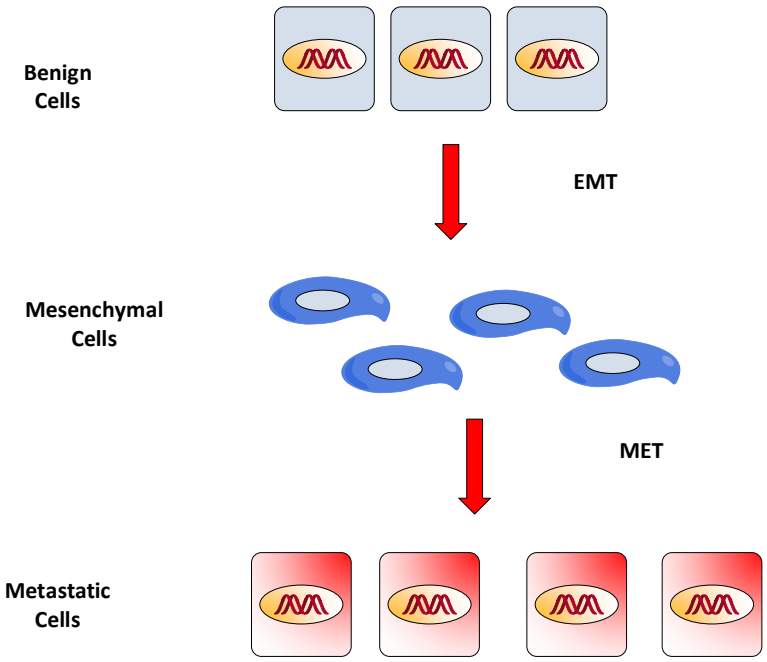


2.4 TOTALITY IN PCA

In the paper by Shtivelman et the authors present the configuration below for PCa where they show end states as EMT, namely loss of position control, and the internal Androgen Receptor activity and the collection of genes in the totality of the control path.



Basically there is a fundamental factor in EMT processes in cancer, Namely the organized mass of cells, change via EMT to moveable cells but when located in a place where they can grow the cells reorganize via a MET transformation. We graphically show this below. This interesting characteristic means that cells that are malignant epithelial like a the original location after being metastasized become agglomerate epithelial like at another. Melanoma is a typical example of this effect


Benign Cells
EMT
Mesenchymal Cells
MET
Metastatic Cells

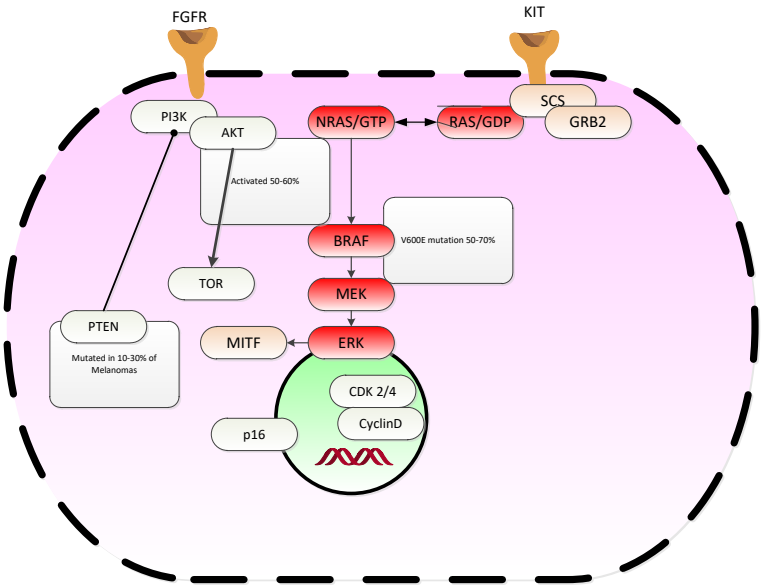
3 WNT PATHWAY AND E-CADHERIN

In this appendix we consider the Wnt pathway, the effects of extracellular signals and the resultant pathway activation. As we indicated earlier ERG is part of the ETS family which is activated by MAP kinase pathways. We show herein the extent of such activation.

3.1 EXTRACELLULAR FACTORS AND MELANOMA: WNT AND E-CADHERIN

Cell growth and cell movement are two characteristics of cancer. We examine this from two perspectives, one for melanoma and another for the benign condition called Dupuytren's disease which is a genetically related disease of excess growth of the fascia in the hands. Both are controlled by the Wnt gene product and both have a relationship with E cadherin which is a protein on the surface of the cell which causes adhesion of cells to cells. Also both Wnt and cadherin are extracellular. Namely Wnt flows between the cells and attaches to certain ligands and then if the intracellular elements are properly aligned the cell starts to proliferate. E cadherin is a surface protein which binds the cell to a certain location. When E cadherin fails then we see the cell start to move from where it is supposed to stay. Thus these two factors present a small picture on the loss of control which we observe in cancers.

The key issue is to understand pathways and points at which they break and where they can be controlled as shown below:



Although the above diagram deals not with Wnt or E Cadherin but the more classic sets of pathway elements we can see how progress can be made. PTEN for example is common in many cancers. We will discuss this at length later. It is seen somewhat in melanoma as well. BRAF is a major control point for melanoma and most of the recent work in pathway control has been focused here. It is possible to block this point which when the mutation occurs and it fails to

control the cell reproduction we see uncontrolled growth. This type of model we will use over and over again.

Thus in the section we look briefly at two key characteristics of cancer, uncontrolled replication and uncontrolled movement, and examine two related mechanisms which control both actions. There will be many others we will introduce. These are useful as examples.

3.2 WNT

Wnt is a gene product which acts by adhering to surface proteins on the outside of a cell. By so adhering, the Wnt can then influence the internal pathways in the cell often blocking apoptosis and initiating a cell growth and proliferation. In simple terms Wnt activates a pathway that allows β-catenin to enter the nucleus and activate a set of transcription promoters which in turn start the process of cell growth and proliferation.

The following is a typical Wnt pathway action:

1. Wnt is generated in a cell and is then secreted and in turn moves extra-cellularly to bind as a ligand on other cell surface receptors.

2. Wnt binds to the cell surface receptor Frizzled and it results in the activation of that receptor.

3. Activated Frizzled inhibits GSK-3 by means of the Disheveled protein. GSK-3 (Glycogen synthase kinase) normally inhibits β-catenin. This is a critical step because once activated β-catenin will result in a cascade of other actions resulting in cell growth. GSK normally activated phosphorylates β-catenin to keep it inactivated..

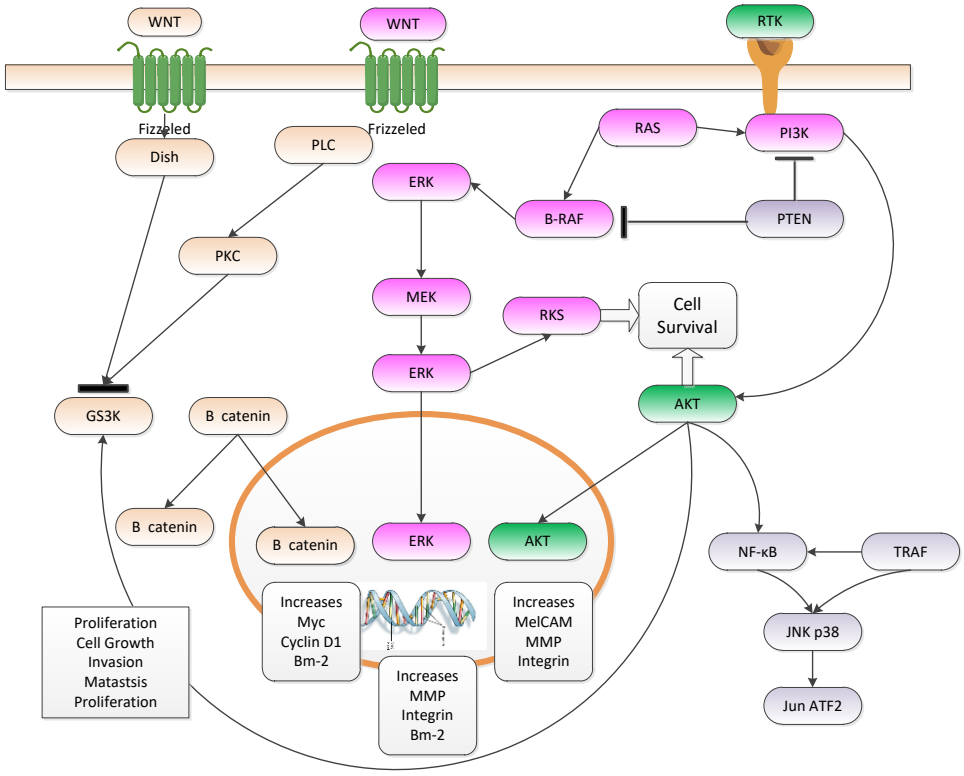
4. β-catenin now accumulates in the cytoplasm and at a certain concentration level β-catenin is transported into the nucleus.

5 When β-catenin is in the nucleus it activates TCF/LEF, a protein which is a transcription factor, and combined these results in the transcription of MYC, a strong proto-oncogene as well as CCDN1.

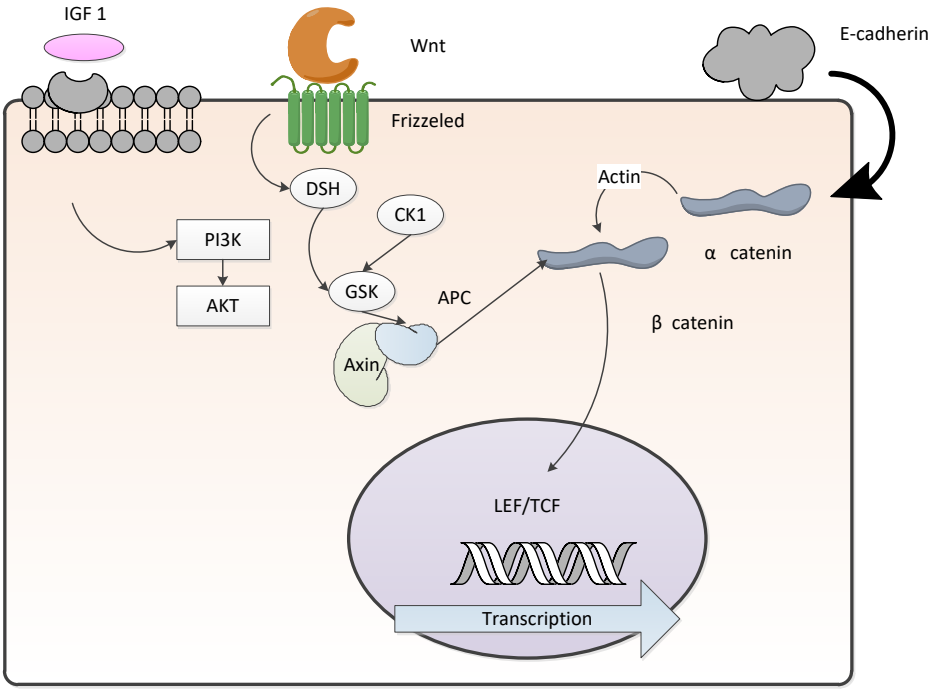
6. This results in uncontrolled cell growth and proliferation.

This is but one of many such pathways but one which is surface activated.

In the graphic below we depict some of the complex pathway processes and their effects. We show the catenin, ERK and Akt effects as each of their control mechanisms are affected. These are three of the major pathway challenges to normal cell homeostasis.



E cadherin is a set of molecules which are attached to the surface of cells and act in a manner to affect cell to cell adhesion. For example a melanocyte adheres to a keratinocyte in the basement membrane of the skin. If this adhesion fails for some reason then the melanocyte can start to wander off. When that happens and the melanocyte moves upward to the epidermis away from the basement layer we call that a melanoma in situ. The cells may not have yet gained the ability to reproduce in excess but they have lost a key element of a health melanocyte, namely the ability to stay fixed. In fact as we shall discuss the E cadherin is replaced by an N cadherin which often allows proliferating melanocytes to cluster together in groups and not have the simple keratinocyte structure.



As noted by Cavallaro and Christofori:

As well as their crucial role in assembling the E-cadherin- mediated cell-adhesion complex, β-catenin and γ-catenin also have important functions in the canonical WNT signalling pathway. Non-sequestered, free β-catenin and γ-catenin are rapidly phosphorylated by glycogen synthase kinase 3β (GSK-3β) in the adenomatous polyposis coli (APC)–axin–GSK-3β complex and are subsequently degraded by the ubiquitin–proteasome pathway.

If the tumour suppressor APC is non-functional, as in many colon cancer cells, or if GSK-3β activity is blocked by the activated WNT-signalling pathway, β-catenin accumulates at high levels in the cytoplasm.

Subsequently, it translocates to the nucleus, where it binds to members of the TCF/LEF1 family of transcription factors and modulates the expression of their target genes, including c-MYC, cyclin D1, fibronectin, MMP7, ID2, CD44, NrCAM, axin-2 (conductin), TCF1 and others, which are mostly genes implicated in cell proliferation and tumour progression. This dual function of β-catenin has motivated several experiments to address whether the loss of E-cadherin function would subsequently lead to the activation of the WNT signalling pathway.

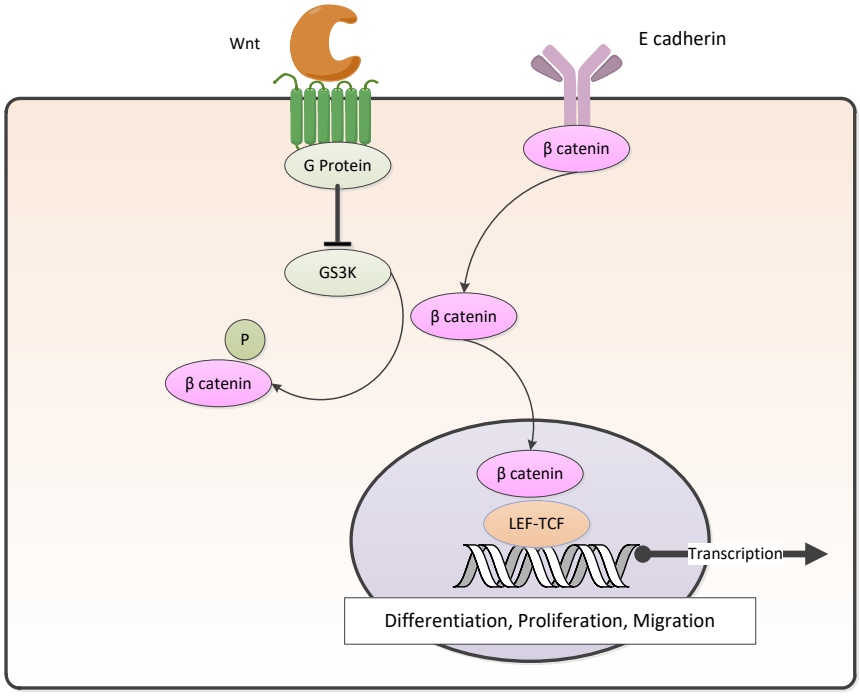
We demonstrate some of this detail below. The catenin is attached to the E-cadherin which is released and if Wnt is activated then the GSK3 is blocked and it migrates to the nucleus where it induces cell proliferation.

Now Wnt is a major component in many cells and especially those that are required for reproduction like bone marrow, colon cells, and skin cells. The control of the Wnt process is essential for homeostasis. Clearly one would want to have cells requiring proliferation to have a well-regulated Wnt path. Thus keratinocytes need continual proliferation since the move from the basement membrane to the skin surface where they are sloughed away. However this would not be the case for melanocytes, where we need limited control. Melanocytes generally remain fixed at the basement membrane and movement or proliferation is inhibited.

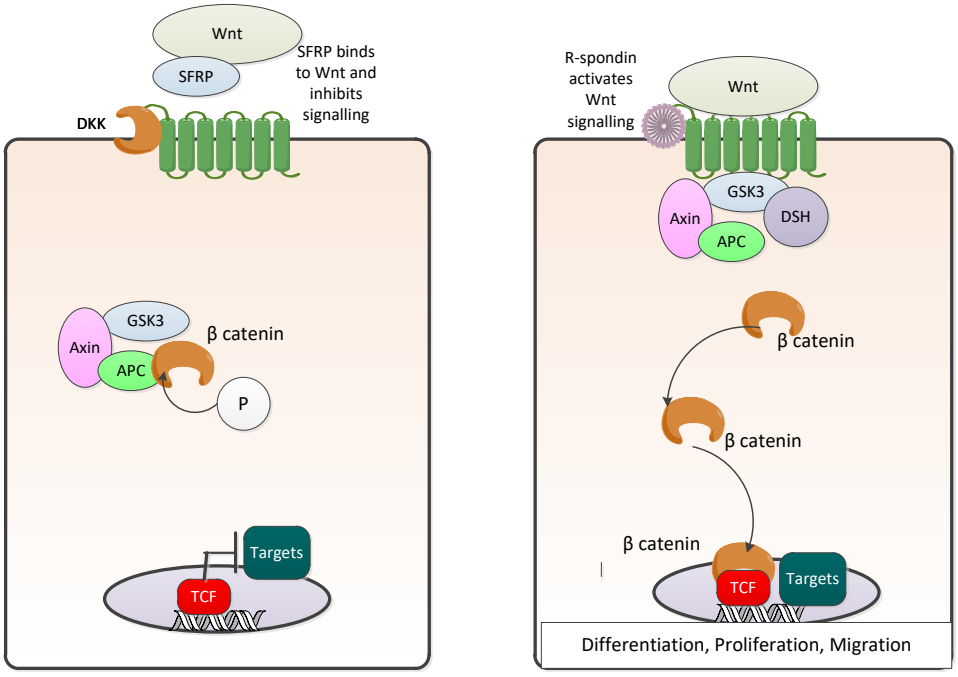
The authors continue:

*In several human cancer types, including **melanoma, prostate and breast cancer**, loss of E-cadherin function is accompanied by de novo expression of mesenchymal cadherins, such as N-cadherin and cadherin-11 (OB-cadherin. Cadherin-11 is expressed in invasive breast cancer and in breast cancer cell lines, and a carboxy-terminally truncated, alternatively spliced form of cadherin-11 can induce an invasive phenotype even in E-cadherin-positive breast cancer cell line. Upregulated expression of P-cadherin in breast cancers and of cadherin-6 in renal cell carcinoma also correlates with poor prognosis.*

By contrast, T-cadherin (also known as H-cadherin) behaves more like E-cadherin: it is downregulated in basal and squamous-cell carcinomas of the skin, correlating with an invasive phenotype. N-cadherin has been shown to promote cell motility and migration — an opposite effect to that of E-cadherin.



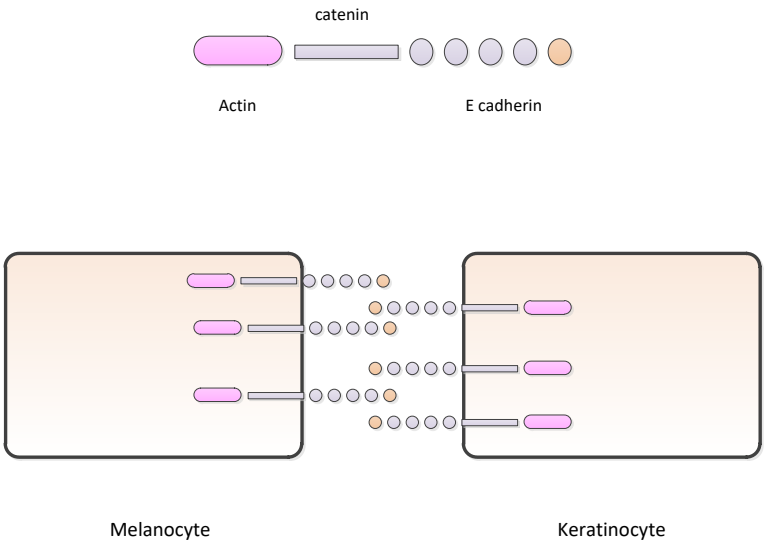
Adapted from Miller and Mihm NEJM July 2006



Modified from http://www.nejm.org/doi/full/10.1056/NEJMoa1101029#t=article

3.3 E CADHERIN

We now examine the cadherin structure. This we depict below as the bonding of two cells via the E cadherin elements which themselves are attached to a catenin and actin proteins within the cell wall. The E cadherin is a bonding/binding protein which finds other specific bonding proteins and then attaches itself within a specified framework. Thus in the basement layer of the skin, at the bottom of the epidermis, the melanocyte attaches uniquely to a keratinocyte and fixes its position in the basement layer so as not to migrate.



E-cadherin is generated at 16q21.1. As is stated in MMMP[5]:

E-cadherin is one of the most important molecules of cell-cell adhesion in non-neural epithelial tissues. This 120 kDa transmembrane glycoprotein is generally localized on the surface of epithelial cells in a region of cell-cell contact that is known as the adherens junction. Cadherins are calcium-dependent cell adhesion proteins. They preferentially interact with themselves in a homophilic manner in connecting cells: cadherins may thus contribute to the sorting of heterogeneous cell types. CDH1 is involved in mechanisms regulating cell-cell adhesions, mobility and proliferation of epithelial cells. It is a ligand for integrin alpha-E/beta-7. Acts as a disulfide-linked homodimer. Interacts directly (via the cytoplasmic domain) with CTNNB1 or JUP to form the PSEN1/cadherin/catenin adhesion complex which connects to the actin skeleton through the actin binding of alpha-catenin.

Interaction with PSEN1 cleaves CDH1 resulting in the disassociation of cadherin-based adherens junctions (CAJ). Anchored to actin microfilaments through association with alpha-catenin, beta-catenin and gamma-catenin. Sequential proteolysis induced by apoptosis or calcium influx, results in translocation from sites of cell-cell contact to the cytoplasm.

During apoptosis or with calcium influx, cleaved by a membrane-bound metalloproteinase (ADAM10), PS1/gamma-secretase and caspase-3 to produce fragments of about 38 kDa (E-CAD/CTF1), 33 kDa (E-CAD/CTF2) and 29 kDa (E-CAD/CTF3), respectively. Processing by the metalloproteinase, induced by calcium influx, causes disruption of cell-cell adhesion and the subsequent release of beta-catenin into the cytoplasm. The residual membrane-tethered cleavage product is rapidly degraded via an intracellular proteolytic pathway. Cleavage by caspase-3 releases the cytoplasmic tail resulting in disintegration of the actin microfilament system. The gamma-secretase-mediated cleavage promotes disassembly of adherens junctions.

In fact the β catenin is bound to the tail of the E cadherin complex and it is released when Wnt is activated. It is this release and movement to the nucleus which gives rise to proliferation.

[5] http://www.mmmp.org/MMMP/public/biocard/viewBiocard.mmmp?id=1301

3.4 WNT, E-CADHERIN AND DUPUTRYEN'S DISEASE

Duputryen's disease is a benign disorder of excess clustered nodular growth of the fibrin, and is a benign fibromatosis of the hand (see Dolmans et al and see Tubiana et al) . What seems to happen is the cells of fibroblasts are overly activated and bind together in clumps creating nodule like structures which then impinge on nerves and muscles impeding hand motion.

As Dolmans et al state:

The WNT gene family consists of structurally related genes that encode glycoproteins, extracellular signaling molecules. Abnormal Wnt signaling is linked to a range of diseases, especially cancer. The best-understood Wnt-signaling pathway is the canonical pathway, which activates the nuclear functions of β-catenin, leading to changes in gene expression that influence cell proliferation and survival.[18] Abnormal proliferation of fibroblasts is a key feature in the early development of Dupuytren's disease. The disease can be divided into three histologic stages:

stage 1, proliferation of fibroblasts;

stage 2, differentiation of fibroblasts into myofibroblasts; and

stage 3, formation of mature type 1 collagen.

Wnt signaling is known to regulate the proliferation and differentiation of fibroblasts in both cancer and fibromatosis. Most of our knowledge of Wnt signaling is derived from studies of cancer. In colon cancer, up-regulation of Wnt signaling causes intestinal crypt cells to proliferate for longer than usual before they migrate and differentiate. This prolonged proliferation phase results in the formation of polyps and confers a predisposition to cancer.

There is substantial evidence to show that this up regulation leading to proliferation is common in prostate cancer and melanoma as well.

They continue:

The Wnt proteins Wnt2, Wnt4, and Wnt7B, which were identified on GRAIL analysis, bind to frizzled receptors, leading to a cascade of events that eventually result in a decrease in the rate of β-catenin degradation. Secreted frizzled-related proteins, such as SFRP4, antagonize the Wnt-signaling pathway by binding to either Wnts or frizzled receptors, thereby affecting receptor occupancy. In the absence of active Wnt, β-catenin is degraded, and potential target genes will not be activated.

We depict the signalling below. Note in the inhibited case we have an extracellular SFRP binding to Wnt and preventing it from activating the pathway as a ligand. Also not that what is driving this is Wnt 2,4 and 7B. Melanoma id driven by Wnt 5.

3.5 BRAF AND WNT

Recently we have seen targeted drugs to control BRAF.

In the paper by Biechele et al we have:

Because the Wnt/β-catenin signaling pathway is linked to melanoma pathogenesis and to patient survival, we conducted a kinome small interfering RNA (siRNA) screen in melanoma cells to expand our understanding of the kinases that regulate this pathway. We found that BRAF signaling, which is constitutively activated in many melanomas by the BRAF(V600E) mutation, inhibits Wnt/β-catenin signaling in human melanoma cells.

Because inhibitors of BRAF(V600E) show promise in ongoing clinical trials, we investigated whether altering Wnt/β-catenin signaling might enhance the efficacy of the BRAF(V600E) inhibitor PLX4720. We found that endogenous β-catenin was required for PLX4720-induced apoptosis of melanoma cells and that activation of Wnt/β-catenin signaling synergized with PLX4720 to decrease tumor growth in vivo and to increase apoptosis in vitro. This synergistic enhancement of apoptosis correlated with reduced abundance of an endogenous negative regulator of β-catenin, AXIN1. In support of the hypothesis that AXIN1 is a mediator rather than a marker of apoptosis, siRNA directed against AXIN1 rendered resistant melanoma cell lines susceptible to apoptosis in response to treatment with a BRAF(V600E) inhibitor.

Thus, Wnt/β-catenin signaling and AXIN1 may regulate the efficacy of inhibitors of BRAF(V600E), suggesting that manipulation of the Wnt/β-catenin pathway could be combined with BRAF inhibitors to treat melanoma.

We now know that if a person has the BRAF V600E presence that use of inhibitors will manage the melanoma for a period.

4 PCA

Prostate cancer is a classic model for a glandular cancer akin to many others such as breast and even thyroid[6]. The prostate gland is composed of a complex matrix of smaller glands with basal and luminal cells and a matrix which has a collection of neuroendocrine cells. As Mulholland et al note:

Epithelial to mesenchymal transition (EMT) is a vital process for morphogenesis during embryonic development, but more recently it has also been implicated in the conversion of early stage tumors into invasive malignancies. Progression of most carcinomas toward malignancy is associated with the loss of epithelial differentiation and by switching toward mesenchymal phenotype, which is accompanied by increased cell motility and invasion. Recent studies have demonstrated that EMT plays a critical role not only in tumor metastasis but also in tumor recurrence that is believed to be tightly linked with the biology of cancer stem-like cells or cancer-initiating cells.

However, the mechanisms by which EMT cells generate the stem-like cells remain to be elucidated. MicroRNAs (miRNAs) are emerging as master regulators of cell differentiation and involved in the acquisition of EMT phenotype during tumor progression. Two evolutionary conserved families, miR-200 and let-7 have been shown to regulate the differentiation processes during the development.

Interestingly, recent studies have also shown that miR-200 family not only could regulate the processes of EMT by targeting E-box binding protein ZEB1 and ZEB2 but was also associated with stem-like cell signatures by regulating the expression of Bmi1 …In summary, the findings reported in this study showed, for the first time, that ARCaPM and PC3 PDGF-D cells having EMT phenotype shared cellular and molecular characteristics of stem cells or cancer stem-like cells. Moreover, miR-200 and let-7 played a critical role in linking EMT phenotype with stem cell signatures by regulating the expression of Lin28B and Notch1.

Therefore, we believe that these models would be useful in screening drug libraries for finding newer agents that could be useful in selective killing of EMT-type or cancer stem-like cells in prostate cancer in the future consistent with a similar approach that showed a great success in breast cancer

Now Shtivelman et al have presented significant results regarding EMT and prostate cancers. They note:

In contrast to its barrier role during cancer initiation, TGFβ promotes metastatic phenotype in late stages by driving epithelial mesenchymal transition. TGFβ and TGFβR are expressed at higher levels in metastatic PCa, and are instrumental in EMT that is mediated, in part, by upregulation of the molecular chaperone clusterin via EMT transcription factor TWIST1. While SMAD3 contributes to activation of AR transcriptional activity, SMAD4, together with SMAD3

[6] See McGarty, Prostate Cancer Genomics, http://www.telmarc.com/Documents/Books/Prostate%20Cancer%20Systems%20Approach%2003.pdf

can also interact with AR and repress AR mediated transcription…A recent study suggests that metastasis suppressor p63 inhibits EMT and metastases at least in part via regulation of miR-205. Either or both p63 and miR-205 are absent in lymph nodes or distant metastases of PCa patients…ERβ is downregulated in high grade PCa via TGFβ and hypoxia, and loss of ERβ is sufficient to promote EMT in PCa.

They continue:

EMT endows cells with migratory and invasive properties, induces stem cell properties, and prevents apoptosis and senescence, thus orchestrating the initiation of metastasis.

EMT is characterized by the loss of expression of E-cadherin and induction of N-cadherin, loss of cell polarity and dependence on adhesion, all contributing to metastatic phenotype. Numerous pathways have been implicated in EMT in PCa, including some developmental pathways, inflammation driven signaling, ERG fusions and others, some of which are listed below. Androgen deprivation induces expression of N-cadherin and EMT in vitro and in patients.

This transition was observed in normal prostate upon ADT and in PCa patients treated with ADT, and involves transcription factor ZEB1. In addition, upregulation of ZEB proteins is induced by several growth factors such as IGF-1 and PDGF-β that promote EMT in vitro.

EZH2 can induce EMT and increase the metastatic potential of prostate cancer cells by downregulation of DAB2IP, a tumor-suppressive Ras GTPase-activating protein (RasGAP. EZH2 is, in turn, regulated by SOX4 a homeobox transcription factor that was shown to act as an oncogene in PCa based on its overexpression and essential role in survival of PCA in vitro [285]. SOX4 appears to be a master regulator of EMT primarily through upregulating EZH2 expression in breast cancer.

TMPRSS2/ERG was also shown to promote EMT via direct transcriptional activation of expression of ZEB1, and indirect activation of ZEB2 through IL1R2 and SPINT1. In addition, ERG induces loss of cell adhesion by activating the WNT pathways through FZD4 to induce EMT and loss of cell adhesion. TGF-β represents a potent EMT inducer in normal development and tumor progression via Smad-dependent and independent transcriptional pathways. Smad mediated induction of Snail, Slug, and Twist via high motility group A2 (HMGA2) and Smad-independent phosphorylation of Par6 contribute to dissolution of cell junction complexes.

TGF-β also induces expression of clusterin, a pleotropic chaperone protein through activation of TWIST1, a known inducer of EMT. Interestingly, another chaperone protein HSP90, in its secreted form, was shown to be involved in EMT of PCa cells in vitro and in patients. TWIST1 is upregulated by enzalutamide treatment along with activation of PKC, and both could be reversed by addition of PKC inhibitor Ro31-8220, at least in vitro, suggesting a potential approach to overcoming EMT associated with androgen deprivation.

5 MELANOMA

Melanoma is considered one of the most aggressive cancers[7]. Recently there has been great progress in immunotherapeutic approaches as well as kinase inhibiting approaches to mitigating the metastatic effects. However it is an interesting cancer in that it can be detected when a CIS (carcinoma in situ) where the cells have lost adhesion to the bottom of the top layer of the skin, just above the basal layer. E cadherin breaks down and the cell stops its quasi epithelial behavior and moves. It is this movement rather than proliferation that makes it a CIS and not a malignant melanoma. It is not fully clear if the CIS will always become a full melanoma, superficial or even nodular. But being where it is not to be is a concern.

As Fenouille et al have noted:

Epithelial to mesenchymal transition (EMT) is a highly conserved developmental program activated during mesoderm formation and neural crest development. This program has also been implicated in promoting dissemination of single malignant cells from primary epithelial tumors. During EMT, cells discard their epithelial characteristics, including cell adhesion and polarity, reorganize their cytoskeleton and acquire a mesenchymal morphology and the ability to migrate. One of the hallmarks of EMT is the functional loss of the cell-cell junction protein Ecadherin. E-cadherin is considered a suppressor of tumor invasion and consistently, loss or partial loss of E-cadherin has been associated with metastatic dissemination and poor prognosis in several solid tumors.

Several transcription factors have been identified that can repress E-cadherin expression including SNAIL/SNAI1, SLUG/SNAI2, ZEB1, ZEB2/SIP1, Twist proteins and E47. These EMT transcription factors bind to E-box elements in the promoter region of E-cadherin leading to transcriptional repression of junctional complexes and induction of the mesenchymal phenotype. Cutaneous melanoma is an aggressive and potentially fatal form of cancer that derives from melanin-producing melanocytes in the epidermis. Melanocytes originate in the neural crest, a population of highly migratory embryonic cells .

Melanoma is a neoplasm of neuroectodermal origin and because of this melanoma cells may not undergo classic EMT-like changes. However, their ability to invade into the dermis is associated with an EMT-like phenotype characterized by changes in expression of cell-cell adhesion molecules of the cadherins family. In normal skin, E-cadherin mediates contacts between melanocyte and adjacent keratinocytes.

During melanoma progression, the transition from radial growth phase (RGP) to invasive or vertical growth phase (VGP) is characterized by decreased E-cadherin expression that results in the loss of keratinocyte-mediated growth and motility control. In addition to the loss of E-cadherin, downregulation of other members of classical cadherins such as P- or H-cadherin as

[7] See McGarty, Melanoma Genomics, http://www.telmarc.com/Documents/Books/Melanoma%20Genomics%2007.pdf

well as generation of a truncated secreted form of P-cadherin are frequently observed during progression of melanomas.

As Gong et al have recently noted:

Epithelial-Mesenchymal Transition (EMT) is a critical step in the progression of cancer. Malignant melanoma, a cancer developed from pigmented melanocytes, metastasizes through an EMT-like process. Ten-eleven translocation (TET) enzymes, catalyzing the conversion of 5-methylcytosine (5mC) to 5-hydroxylmethylcytosine (5-hmC), are down regulated in melanoma. However, their roles in the progression and the EMT-like process of melanoma are not fully understood.

Here we report that DNA methylation induced silencing of TET2 and TET3 are responsible for the EMT like process and the metastasis of melanoma. TET2 and TET3 are down regulated in the TGF-β1-induced EMT-like process, and the knocking down of TET2 or TET3 induced this EMT-like process.

A DNA demethylating agent antagonized the TGF-β- induced suppression of TET2 and TET3. Furthermore, a ChIP analysis indicated that enhanced recruitment of DNMT3A (DNA Methyltransferase 3A) is the mechanism by which TGF-β induces the silencing of TET2 and TET3. Finally, the overexpression of the TET2 C-terminal sequence partially rescues the TGF-β1-induced EMT-like process in vitro and inhibits tumor growth and metastasis in vivo. Hence, our data suggest an epigenetic circuitry that mediates the EMT activated by TGF-β. As an effector, DNMT3A senses the TGF-β signal and silences TET2 and TET3 promoters to induce the EMT-like process and metastasis in melanoma.

This identifies many of the steps in the EMT process in melanoma.

6 OTHER CANCERS

We continue with a brief summary of other cancers.

6.1 PANCREATIC

Cancer of the pancreas has always been a significant challenge and the EMT process plays a key role here. As Heerboth et al note:

Pancreatic cancer generally has a poor prognosis, in part because symptoms often do not appear until the cancer is too advanced for surgical treatment. Pancreatic exocrine tumors have an average 5 year survival of up to 14%. Neuroendocrine tumors have a 61% 5-year survival rate if detected at Stage 1, but these tumors are rarely detected at this phase. Thus, early detection and inhibition of metastasis remain among the greatest challenges in the treatment of these tumors.

Several genes related to EMT have been considered with respect to these clinical challenges. In one in vitro study, Hh inhibition with cyclopamine resulted in down-regulation of Snail and up-regulation of E-cadherin, as well as a striking reduction of invasive capacity. Combining gemcitabine and cyclopamine completely abrogated metastasis while also significantly reducing the size of "primary" tumors. These findings suggest that inhibition of the Hh pathway is a valid therapeutic strategy for pancreatic cancer that particularly targets metastasis.

As Song et al note:

FOXA1 and FOXA2, members of the forkhead transcription factor family, are critical for epithelial differentiation in many endoderm-derived organs, including the pancreas. However, their role in tumor progression is largely unknown. Here, we identified FOXA1 and FOXA2 as important antagonists of the epithelial-to-mesenchymal transition (EMT) in pancreatic ductal adenocarcinoma (PDA) through their positive regulation of E-cadherin and maintenance of the epithelial phenotype. In human PDA samples, FOXA1/2 are expressed in all epithelia from normal to well-differentiated cancer cells, but are lost in undifferentiated cancer cells.

In PDA cell lines, FOXA1/2 expression is consistently suppressed in experimental EMT models and RNAi silencing of FOXA1/2 alone is sufficient to induce EMT. Conversely, ectopic FOXA1/2 expression can potently neutralize several EMT-related E-cadherin repressive mechanisms. Finally, ectopic FOXA2 expression could reactivate E-cadherin expression in a PDA cell line with extensive promoter hypermethylation.

In fact, demethylation mediated reactivation of E-cadherin expression in these cells required concurrent reactivation of endogenous FOXA2 expression. We conclude that suppression of FOXA1/2 expression is both necessary and sufficient for EMT during PDA malignant progression.

The ever present FOXA proteins are significant elements in EMT as well.

6.2 HEPATOCELLULAR

Primary liver cancer is most likely driven by inflammatory factors due to its function in the body. As Heerboth et al note:

Hepatocellular carcinoma (HCC), which is among the most deadly forms of cancers worldwide, is the most common primary liver cancer and is the fastest growing cause of cancer death in men in the United States. The dominant risk factors are chronic Hepatitis B or Hepatitis C infection. In addition, cirrhosis can have an effect on the tumor microenvironment as well as on tumorigenesis.

Cirrhosis can lead to the activation of stellate cells, which increase production of extracellular matrix proteins, cytokines, and growth factors, many of which can alter hepatocyte proliferation and promote tumorigenesis. HCC tends to have a poor prognosis due to late diagnoses and a lack of effective treatment options.

While EGFR-targeted therapies have been successful in some types of cancers, erlotinib and cetuximab have not been very effective in clinical HCC trials, particularly in the treatment of mesenchymal HCC cells. In the case of hepatic carcinomas, Sorafenib, which inhibits STAT3 and phosphorylates TGF-β which are both up-regulated in EMT, is also being studied as a potential therapeutic agent

Metastatic spread is enhanced by the accumulation of additional blood flows. As Jue et al noted:

Hypervascularity is one of the main characteristics of hepatocellular carcinoma (HCC). However, the mechanisms of angiogenesis in HCC remain controversial. In this study, we investigate the role of Notch1 in angiogenesis of HCC.

We found that Notch1 expression was correlated with formation of vasculogenic mimicry (VM) and expression of biomarkers of epithelial-to-mesenchymal transition (EMT) in the tumor specimens. Two HCC cell lines, HepG2 and MHCC97-H, with low and high Notch1 expression, respectively, were used to study the mechanism of VM formation both in vitro and in vivo. It was found that MHCC97-H cells, but not HepG2 cells form VM when they grow on matrigel in vitro. HepG2 cells gained the power of forming VM when they were overexpressed with Notch1, while knockdown Notch1 expression in MHCC97-H cells led to the loss of VM forming ability of the cells.

Similar results were found in in vivo study. High expression of Notch1 in HepG2 promoted xenograft growth in nude mice, with abundant VM formation in the tumor samples. Moreover, we observed Notch1 was associated with the EMT and malignant behavior of hepatocellular carcinoma by analyzing clinical specimens, models for in vitro and in vivo experiments.

HepG2 presented EMT phenomenon when induced by TGF-β1, accompanied by Notch1 activation while MHCC97-H with knockdown of Notch1 lost the responsiveness to TGF-β1

induction. Our results suggest that Notch1 promotes HCC progression through activating EMT pathway and forming VM. Our results will guide targeting Notch1 in new drug development.

6.3 COLORECTAL

The digestive system and especially the colon present significant opportunities for malignant transformation due to the ever present sets of challenges from environmental assaults. As Heerboth et al note:

Colorectal cancers tend to start as a small growth in the inner lining of the colon known as a polyp, ultimately giving rise to adenocarcinomas. Colorectal cancer is one of the most common cancers, and yet it is not among the most lethal cancers as early clinical detection via routine screenings has dramatically improved overall mortality. Still, careful study of EMT markers has revealed additional clinically relevant information. A clear link has been established between CD44, enhancement of EMT, and colon cancer invasion.

Furthermore, FGFR4 has also been shown to play a crucial role in tumorigenesis, invasion, and survival in colorectal cancer, and its specific targeting marks a new avenue of colorectal cancer therapy. Vimentin is highly expressed in the stroma of colorectal cancer cells compared to healthy cells, but interestingly, not in the cancer cells themselves. Higher levels of stromal vimentin have been correlated with poor prognosis of colorectal cancer. Specifically, since vimentin is expressed in mesenchymal cells and not epithelial cells, it indicates that EMT has taken place

The Vogelstein paradigm seems to hold true in the colon although it could be challenged elsewhere.

6.4 PULMONARY

Lung cancer has been closely tied to smoking cigarettes yet even as smoking, especially in men, has been reduced, there still is a significant number of new cases of both small and large cell.

As Shih and Yang noted:

Lung cancer is the leading cause of cancer death worldwide. Cancer metastasis and resistance to treatment (including radiotherapy, chemotherapy and targeted therapy) are two major causes for the poor survival of lung cancer patients. Epithelial– mesenchymal transition (EMT) is involved in cancer cell invasion, resistance to apoptosis and stem cell features. The process of EMT is controlled by a group of transcriptional factors, zinc finger proteins and basic helix-loop-helix factors.

Signaling pathways activated by intrinsic or extrinsic stimuli converge on these transcriptional factors and regulated the phenotypic changes of cancer cells. These EMT regulators may play an important role in cancer progression. In lung cancer, Slug is the most thoroughly investigated EMT regulator. The expression of Slug is associated with lung cancer invasion and resistance to

target therapy. In this review, we focus on the current understanding of the role of Slug in the carcinogenesis and progression of lung cancer.

Specifically as Heerboth et al note:

Adenocarcinoma is a type of cancerous tumor that forms from glandular structures. Stromal periostin protein is associated with versican collagen, and tumor cell epithelial periostin is associated with both versican and vimentin.

Each of these associations suggests that cancer cells have undergone EMT and become more metastatic, but surprisingly, this study did not find a correlation between vimentin up-regulation and morphological trans-differentiation. However, the authors observed that the up-regulation of stromal vimentin, periostin, and versican is associated with higher cancer grades. As vimentin is the constituent of the cytoskeleton network, it is possible that stromal populations go through certain changes during the induction of EMT. Similar results were found in breast carcinoma

6.5 BREAST

In many ways breast cancer is similar to prostate cancer. It is primarily a glandular cancer although there are variants. As Takahashi et al note:

Epithelial-to-mesenchymal transition (EMT) is an evolutionarily conserved process that occurs during embryonic development in many species of mammals. Since the EMT program is often activated during tumor invasion and metastasis, the genetic controls and biochemical mechanisms underlying the acquisition of invasiveness and the subsequent systemic spread of cancer cells have been areas of intensive research.

The EMT phenotype is characterized by the downregulation of epithelial markers such as E-cadherin, the expression of mesenchymal markers such as N-cadherin and vimentin, the loss of cell-cell contact and cell polarity, and the acquisition of cell invasive capabilities. …EMT is also associated with the acquisition of CSC properties. A $CD44^{+}/CD24^{-/low}$ cell population purified from cancer tissues shows the features of an EMT phenotype, and human cancer cells induced to undergo EMT exhibit a $CD44^{+}/CD24^{-/low}$ antigen phenotype and high tumorigenicity.

Thus EMT clearly plays a role in a wide variety of these cancers.

7 IMMUNE FACTORS

A question can be posed regarding the transition of epidermal cells to mesenchymal. Namely; can one use the markers associated with transition as targets for some form of immune therapy. As we see with hematopoetic cells, we can use CD-19, some new targets such as CD-30 are also available. Then perhaps one can also develop CAR-T cell approaches, or viral vectors. There are a multiplicity of means and methods dependent however on unique and pervasive targets on the cells themselves[8].

As Uarez-Carmona et al have noted:

During the metastatic progression of epithelial tumors, tumor cells indeed undergo phenotypic changes, essentially driven by environmental stimuli, allowing the tumor cells to adapt to the different microenvironment encountered (adjacent stroma, blood, or colonized organs). Epithelial-to-mesenchymal transition (EMT) appears today as a major actor modulating these phenotypic conversions.

Although two recent studies have revived the debate about the universal requirement of EMT in the metastasis process, the current dogma is that EMT processes might be involved in the initial steps of the metastatic cascade, including tumor invasion, intravasation, and micrometastases formation. This has been supported by multiple in vitro and in vivo functional data, as well as by correlative data in human samples.

The acquisition of EMT-like changes in tumor cells has been extensively studied and implies increased invasive properties, resistance to DNA damage- and chemotherapy-induced apoptosis, immunosuppression, and the acquisition of stem-like features. EMT transcriptomic signatures are found highly associated with groups of patients with poorer outcome in multiple cancer entities including breast cancer, colorectal cancer, head and neck cancer, or malignant pleural mesothelioma.

EMT-associated signaling pathways have lately been considered as therapeutic targets, as recently shown in a murine pancreatic cancer model. In this study, metastasis was successfully hampered by the use of nimbolide, a drug that, among other effects, reduced EMT via the induction of excessive production of reactive oxygen species (ROS). EMT signaling pathways have also been targeted in breast cancer in vitro models, or even in clinical settings …

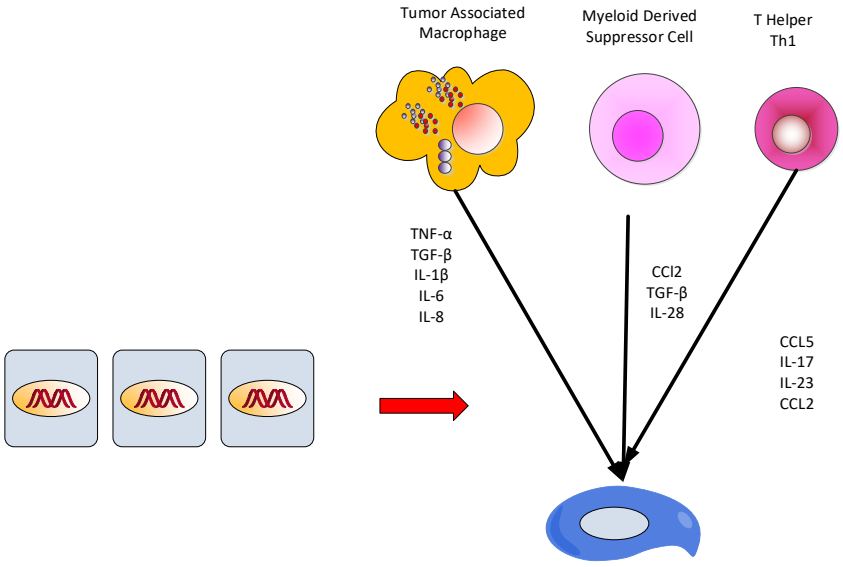
Among immune cells infiltrating solid tumors, TAMs are most likely the most abundant cell type as they make up to 50% of the tumor mass. Even though macrophages should be able to kill tumor cells, provided they get the appropriate activation signals, the chronically inflamed/immunosuppressive microenvironment most often polarizes TAMs into tumor-supporting cells (schematically categorized in 'M2-like macrophages') that promote extracellular matrix remodeling, angiogenesis, immunosuppression, and foster the acquisition of invasive properties by cancer cells by secreting various soluble factors.

[8] See McGarty, Immunotherapy: A Systems Approach, https://www.telmarc.com/Documents/Books/Immune.pdf

The global numbers of TAMs have been correlated with EMT-like features in several cancer entities. This has been reported in a cohort of 178 patients with gastric cancer, where CD68-positive cell density is associated with the expression of EMT features in cancer cells (E-cadherin loss and vimentin de novo expression). This was also demonstrated in an independent study using another marker of TAMs, CD163. In that study, high intratumoral CD163 expression was found to be correlated with E-cadherin loss

Thus some work has been done in this area.

The same authors depict the impacts of immune cells as shown below:



Thus the result of inflammation, especially chronic, can oftentimes result in an EMT and in turn a malignant result. As Radisky had noted:

Mesenchymal cells can contribute to the ECM by synthesizing and organizing new components and by remodeling the ECM through the production of matrix degrading metalloproteinases (MMPs). Mesenchymal cells are also abundant sources of signaling proteins that act on epithelial cells, including growth factors of the epidermal (EGF), hepatocyte (HGF) and fibroblast (FGF) families, as well as transforming growth factor (TGFβ).

Namely, the Mesenchymal calls can become the self-proliferating engines driving cell transformation. Many of these drivers are immune system cytokines and related receptors.

8 OBSERVATIONS

We now consider several observations resulting for the above analysis. We examine four areas:

1. We look at the issue of cancer stem cells and their relationship to the EMT process. CSC are interesting targets of interest since targeting them may be much more effective than targeting bulk tumors. All too often removing a bulk tumor without regard to a CSC presence just means recurrence. It is often the case where a surgeon gets a clear margin on an excision and declares victory while a CSC has escaped.

2. Circulating tumor cells or parts thereof have become of significant interest in what has been termed liquid biopsies. Namely constituents of tumor cells in the blood can be detected and analyzed. Here we look at markers for excess EMT process.

3. There has been an evolving understanding of the EMT process. We briefly discuss this change.

4. The arear of new therapeutics is key. One specific area we have tried to open is based upon the following logic.

a) EMT is related to and a putative driver of metastatic growth.
b) EMT as a process is heavily influenced by immune system drivers
c) Perhaps immunotherapeutic approaches to mitigating EMT processes may be effected and this down regulate any metastatic results.

Thus studying the EMT process can add significantly to our understanding of a multiplicity of cancers.

8.1 STEM CELLS

Stem cells have been discussed at length in the context of many cancers. They are often closely associated with the EMT process. As Mitra et al note:

Tumor relapse and metastasis are the primary causes of poor survival rates in patients with advanced cancer despite successful resection or chemotherapeutic treatment. A primary cause of relapse and metastasis is the persistence of cancer stem cells (CSCs), which are highly resistant to chemotherapy. Although highly efficacious drugs suppressing several subpopulations of CSCs in various tissue-specific cancers are available, recurrence is still common in patients. To find more suitable therapy for relapse, the mechanisms underlying metastasis and drug-resistance associated with relapse-initiating CSCs need to be identified. Recent studies in circulating tumor cells (CTCs) of some cancer patients manifest phenotypes of both CSCs and epithelial-mesenchymal transition (EMT).

These patients are unresponsive to standard chemotherapies and have low progression free survival, suggesting that EMT-positive CTCs are related to co-occur with or transform into relapse-initiating CSCs.

Furthermore, EMT programming in cancer cells enables in the remodeling of extracellular matrix to break the dormancy of relapse-initiating CSCs. In this review, we extensively discuss the association of the EMT program with CTCs and CSCs to characterize a subpopulation of patients prone to relapses.

Identifying the mechanisms by which EMT-transformed CTCs and CSCs initiate relapse could facilitate the development of new or enhanced personalized therapeutic regimens.

We have discussed the CSC construct especially in the case of PCa. It could be argued that identifying the PCa and removing them would then make any of the other cells indolent. CSC development still is a complex area. Just how a CSC is formed and how it manages to survive and prosper is complex. Perhaps the nexus with the EMT process may assist in better understanding.

8.2 CIRCULATING TUMOR CELLS

Circulating Tumor Cells, CTC, and parts therefrom, such as RNA fragments, even DNA fragments, are also a current topic of interest in detecting and monitoring cancers. Since EMT is considered an essential part of the metastatic process, then it would seem logical to also look for EMT markers as well.

As Heerboth et al note:

Another exciting area of research is the use of EMT markers in the analysis of circulating tumor cells (CTC). Diagnostically, CTC has been a mainstay of clinical practice in assessment of metastasis and prognosis. The presence of CTC in a patient's blood can be measured using the AdnaTest, a PCR assay for markers of EMT such as Twist, Akt, and Pi3k. The test employs a method for enriching the CTCs in a blood sample using antibodies conjugated to magnetic beads. Once the tumor cells have been pulled down, the mRNA can be isolated and expression of EMT markers determined. The test is reported to be sensitive enough to detect two CTCs in a 5 mL sample of blood.

Recent works have indicated that consideration of CTC EMT status is critical to achieve a more accurate prognosis. In studies of metastatic breast cancer, CTC were found to express known EMT regulators, including TGF-β pathway components and the FOXC1 transcription factor. These data support a role for EMT in the blood-borne dissemination of human breast cancer. Classical markers of EMT, Twist, and vimentin, have been identified in breast cancer patients and specifically show elevated expression in patients with metastatic cancer relative to patients with early stage cancer, supporting the hypothesis that EMT controls the metastatic potential of CTCs

Thus we see that a more complex set of blood borne markers may be identified and profiled to establish cancer diagnosis, prognosis and arguably even fine tuning on therapeutics and therapeutic targeting.

From Lee et al we have a list of putative markers:

Characteristic	*Targets*
Proteins that increase in abundance	N-cadherin Vimentin Fibronectin Snail 1 (Snail) Snail2 (Slug) Twist Goosecoid FOXC2 Sox10 MMP-2 MMP-3 MMP-9 Integrin avp6
Proteins that decrease in abundance	E-cadherin Desmoplakin Cytokeratin Occludin
Proteins whose activity increases	ILK GSK-3p Rho
Proteins that accumulate in the nucleus	p-catenin Smad-2/3 NF-Kp Snail1 (Snail) Snail2 (Slug) Twist

Whether any of these are specifically appropriate will take time to study. The issue one assumes is to better understand EMT as it pertains to a malignancy. For example, for decades in breast cancer, in melanoma, and other cancers, removal of lymph nodes was considered standard practice even if no overt sign of metastasis was present. The resulting morbidity was often significant. If however one seeks EMT processes then perhaps one may attain a more viable and specific alternative.

8.3 CONTRADICTIONS

There has been a debate over the years regarding the nature of EMT and cancer. As Tian (2005) notes in an earlier paper:

Epithelial mesenchymal transition has been postulated as a versatile mechanism which facilitates cellular repositioning and redeployment during embryonic development, tissue

reconstruction after injury, carcinogenesis, and tumor metastasis. The hypothesis originates from parallels drawn between the morphology and behavior of locomotory and sedentary cells in vitro and in various normal and pathologic processes in vivo.

This review analyzes data from several studies on embryonic development, wound healing, and the pathology of human tumors, including work from our own laboratory, to assess the validity of the proposal. It is concluded that there is no convincing evidence for conversion of epithelial cells into mesenchymal cell lineages in vivo and that the biological repertoire of normal and malignant cells is sufficient to account for the events and processes observed, without needing to invoke radical changes in cell identity.

The author then goes on with a detailed "on the other hand" discussion of EMT relevance. This is always a worthwhile analysis to come back to from time to time.

However Roche (2018) notes some thirteen years later:

The epithelial-to-mesenchymal transition (EMT) occurs during normal embryonic development, tissue regeneration, organ fibrosis, and wound healing. It is a highly dynamic process, by which epithelial cells can convert into a mesenchymal phenotype. However, it is also involved in tumor progression with metastatic expansion, and the generation of tumor cells with stem cell properties that play a major role in resistance to cancer treatment.

EMT is not complete in cancer cells, and tumor cells are in multiple transitional states and express mixed epithelial and mesenchymal genes.

Such hybrid cells in partial EMT can move collectively as clusters, and can be more aggressive than cells with a complete EMT phenotype. EMT is also reversible by the mesenchymal-to-epithelial transition (MET), thought to affect circulating cancer cells when they reach a desirable metastatic niche to develop secondary tumors. The EMT process involves the disruption of cell–cell adhesion and cellular polarity, remodeling of the cytoskeleton, and changes in cell–matrix adhesion. It is associated with improvement in migratory and invasive properties.

In cancers, EMT inducers are hypoxia, cytokines, and growth factors secreted by the tumor microenvironment, stroma crosstalk, metabolic changes, innate and adaptive immune responses, and treatment with antitumor drugs. Switch in gene expression from epithelial to mesenchymal phenotype is triggered by complex regulatory networks involving transcriptional control with SNAI1 and SNAI2, ZEB1 and ZEB2, Twist, and E12/E47 among transcriptional factors, non-coding RNAs (miRNAs and long non-coding RNAs), chromatin remodeling and epigenetic modifications, alternative splicing, post-translational regulation, protein stability, and subcellular localization.

EMT is becoming a target of interest for anticancer therapy. However, more knowledge about the role of EMT in metastasis, its control, and its reversion is necessary. Indeed, alternative modes of dissemination, colonization via a MET-independent pathway, and investigation of circulating cancer cells in the blood support a more nuanced view of the role of EMT and MET in cancer metastasis.

The above argument seems to strengthen the assertion of the significance of EMT and as importantly the MET reversal process which we have discussed.

8.4 THERAPEUTICS

The understanding of the EMT process presents opportunities for therapeutic development. Mladinich et al have noted:

Cancer stem cell (CSC) has become recognized for its role in both tumorigenesis and poor patient prognosis in recent years. Traditional therapeutics are unable to effectively eliminate this group of cells from the bulk population of cancer cells, allowing CSCs to persist posttreatment and thus propagate into secondary tumors. The therapeutic potential of eliminating CSCs, to decrease tumor relapse, has created a demand for identifying mechanisms that directly target and eliminate cancer stem cells. Molecular profiling has shown that cancer cells and tumors that exhibit the CSC phenotype also express genes associated with the epithelial-to-mesenchymal transition (EMT) feature.

Ample evidence has demonstrated that upregulation of master transcription factors (TFs) accounting for the EMT process such as Snail/Slug and Twist can reprogram cancer cells from differentiated to stem-like status. Despite being appealing therapeutic targets for tackling CSCs, pharmacological approaches that directly target EMT-TFs remain impossible. In this review, we will summarize recent advances in the regulation of Snail/Slug and Twist at transcriptional, translational, and posttranslational levels and discuss the clinical implication and application for EMT blockade as a promising strategy for CSC targeting.

Thus there may be avenues of access to controlling the CSC via the EMT process. The authors conclude:

These studies indicate that approaches which inhibit protein expression or activity upstream of EMT-TFs will have a better chance to achieve CSC eradiation. Extensive work as reviewed above shed light on new approaches for the targeting of EMT-TFs. As our understanding of protein regulation of EMT-TFs advances, the ability to generate or repurpose new candidate molecules to target CSCs increases.

Specific inactivation of EMT-TFs in combination with chemotherapy will likely enhance patient survival long-term via targeting of both CSCs and differentiated tumor cells. We have reasons for optimism that future studies on structural information of upstream regulators of EMT-TFs and on the crosstalk between upstream regulators and EMT-TFs would yield new CSC therapeutics.

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