ERG, A MASTER TRANSCRIPTION FACTOR?

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ERG is a transcription factor which can split off and get bound with TMPRSS2. The result is prostate cancer. We examine ERG as a putative Master Transcription Factor, MTF, and as such a target for therapeutics.

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1 INTRODUCTION

Prostate cancer, PCa, is the result a complicated set of genetic changes. Over the years there have been a multiplicity of markers for assessing both the presence and the severity of this malignancy. In addition, there have been many discussions as to which cell dominates, basal or luminal, and also the progression mechanism. Moreover, there has been a long argument of the nexus between HGPIN and PCa, with many arguing that HGPIN is a carcinoma in situ and furthermore it is a prodrome to PCa in all cases. However, as we have shown on multiple occasions this is not always the case. HGPIN is known to abate and disappear totally.

Other factors such as epigenetic actions from methylation, acetylation, miRNAs and the like tend to further confuse the issue.

A question which often arises is; what starts the process off?

Li et al in a recent paper propose that ERG is what they term, "a master transcription factor". Just what such a factor is has been open to some debate. Many researchers have looked for some master control mechanism, a sine qua non of pathway elements. Unfortunately one may end up in a Scholastic debate as to the un-caused cause. I shall leave that issue in the 13th century. However we do know that ERG via a fusion does seem to have a presence in most PCa. As Santos et al have noted:

The model, studied in vitro, demonstrated that prostate epithelial cells, in association with androgen receptor (AR) signaling, develop TMPRSS2-ERG gene fusions when exposed to oxidative stress, contributing to PCa formation. The precise mechanisms by which this occurs are not entirely known, but the researchers proposed that formation of ROS and DNA breaks results from signaling of epithelial cells by inflammatory cytokines, such as tumor necrosis factor (TNF)

ERG is part of the ETS transcription family. We have examined ERG and the fusion implications some seven years ago. ERG is a transcription factor which is considered a proto-oncogene. It can move and fuse proximate to TMPRSS2. PMPRSS2 is a protease gene. This fusion is a common driver in prostate cancers.

Further as Yu et al had noted:

Chromosomal rearrangements fusing the androgen-regulated gene TMPRSS2 to the oncogenic ETS transcription factor ERG occur in approximately 50% of prostate cancers, but how the fusion products regulate prostate cancer remains unclear. Using chromatin immunoprecipitation coupled with massively parallel sequencing, we found that ERG disrupts androgen receptor (AR) signaling by inhibiting AR expression, binding to and inhibiting AR activity at gene-specific loci, and inducing repressive epigenetic programs via direct activation of the H3K27 methyltransferase EZH2, a Polycomb group protein. These findings provide a working model in which TMPRSS2-ERG plays a critical role in cancer progression by disrupting lineage specific differentiation of the prostate and potentiating the EZH2-mediated dedifferentiation program.

In this Note we reexamine ERG in the context of a recent paper by Li[1]. Before doing so we first consider what Shtivelman et al have noted[2]:

Localized PC could be (relatively but not entirely arbitrary) subdivided in two categories based on presence/ absence of TMPRSS2-ERG or other changes in ETS family genes. ETS family fusions are found in up to 60% of PCa, and the fusion-negative group could be divided into several subtypes based on results of the recent NGS studies that have identified new genetic aberrations in this group…

TMPRSS2-ERG fusion is a result of interchromosomal rearrangement that occurs in 40 to 60% of prostate cancers. Other members of ETS family of transcription factors, of which ERG is a member, are also involved in rearrangements, albeit much less frequently. This is the most frequent chromosomal rearrangement found in solid tumors, and perhaps in human cancer in general, considering the high incidence of PCa. Fusions appear to be an early event, found already in PIN, and the presence of TMPRSS2-ERG fusion is thought to be sufficient for the initiation of prostate intraepithelial neoplasia (PIN).

Increased expression of ERG or other ETS factors under control of androgen responsive promoter (TMPRSS2) is an inevitable consequence of the fusion events, and it activates transcriptional program that contributes to oncogenesis by upregulating expression of, among others, MYC, EZH2 and SOX9 and repressing NKX3.1. The net result of high levels of ETS expression is prevention of the differentiation of prostate epithelium that is normally governed by AR. Patients with expression of ERG in high-grade prostatic intraepithelial neoplasia are more likely to develop prostate cancer. Expression of TMPRSS2- ERG fusion shows a striking

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[1] https://www.researchgate.net/publication/315374473_ERG_TMPRSS2_AND_PROSTATE_CANCER

[2] For reference we have https://www.nature.com/scitable/definition/transcription-factor-167/ :

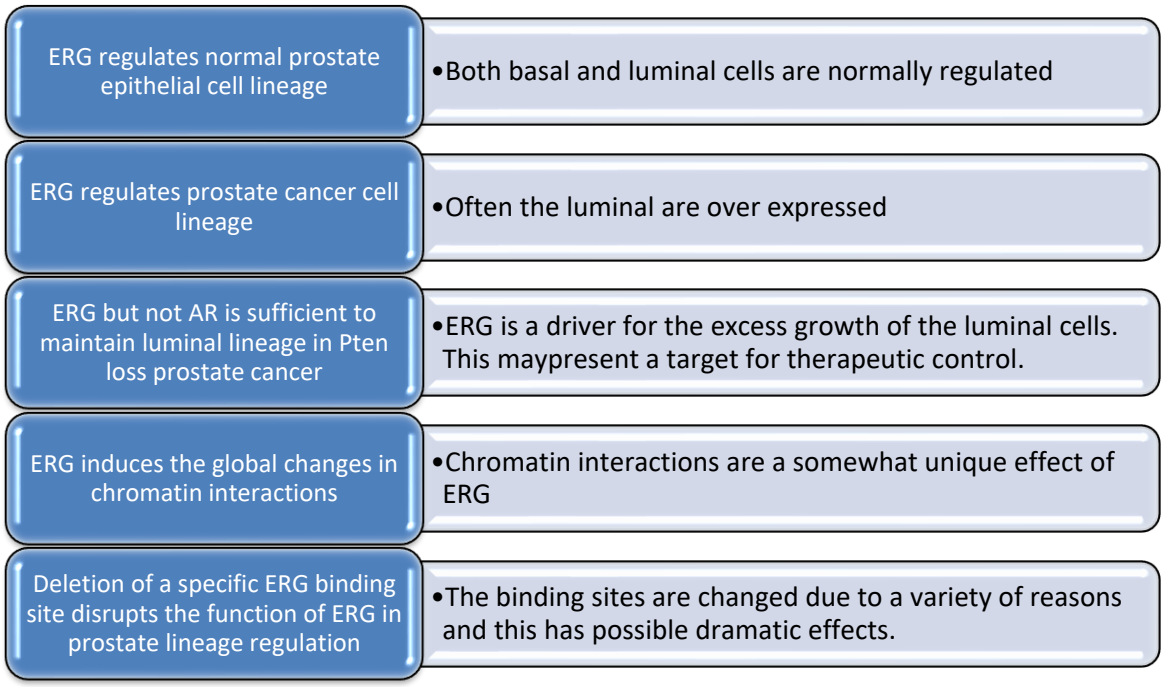
*Promoter sequences are DNA **sequences that define where transcription of a gene by RNA polymerase begins.** Promoter sequences are typically located directly upstream or at the 5' end of the transcription initiation site. RNA polymerase and the necessary transcription factors bind to the promoter sequence and initiate transcription. Promoter sequences define the direction of transcription and indicate which DNA strand will be transcribed; this strand is known as the sense strand. Many eukaryotic genes have a conserved promoter sequence called the TATA box, located 25 to 35 base pairs upstream of the transcription start site. Transcription factors bind to the TATA box and initiate the formation of the RNA polymerase transcription complex, which promotes transcription.*

*Transcription factors are proteins involved in the process of converting, or transcribing, DNA into RNA. Transcription factors include a wide number of proteins, **excluding RNA polymerase,** that **initiate and regulate the transcription of genes**. One distinct feature of transcription factors is that they have DNA-binding domains that give them the **ability to bind to specific sequences of DNA called enhancer or promoter sequences**. Some transcription factors bind to a DNA promoter sequence near the transcription start site and help form the transcription initiation complex. Other transcription factors bind to regulatory sequences, such as enhancer sequences, and can either stimulate or repress transcription of the related gene. These regulatory sequences can be thousands of base pairs upstream or downstream from the gene being transcribed. Regulation of transcription is the most common form of gene control. The action of transcription factors allows for unique expression of each gene in different cell types and during development.*

Note that there are about 1,600 different transcription factors (TF). The ETS family is a significant one but many of the TFs are often active participants in malignancies.

correlation with AR expression in tumor biopsies. It is of significant interest that formation of fusions involving ERG genes has been shown to be facilitated by signaling from the AR, which induces proximity of the TMPRSS2 and ERG genomic loci. Both are located on chromosome 21q22, and fusion occurs via double-stranded DNA breaks.

Now as we have noted, we are considering recent work by Li et al which adds some significant observations to the ERG import. Namely it not only has transcription effects but seems to have certain epigenetic effects as well. The paper by Li asserts the following conclusions:

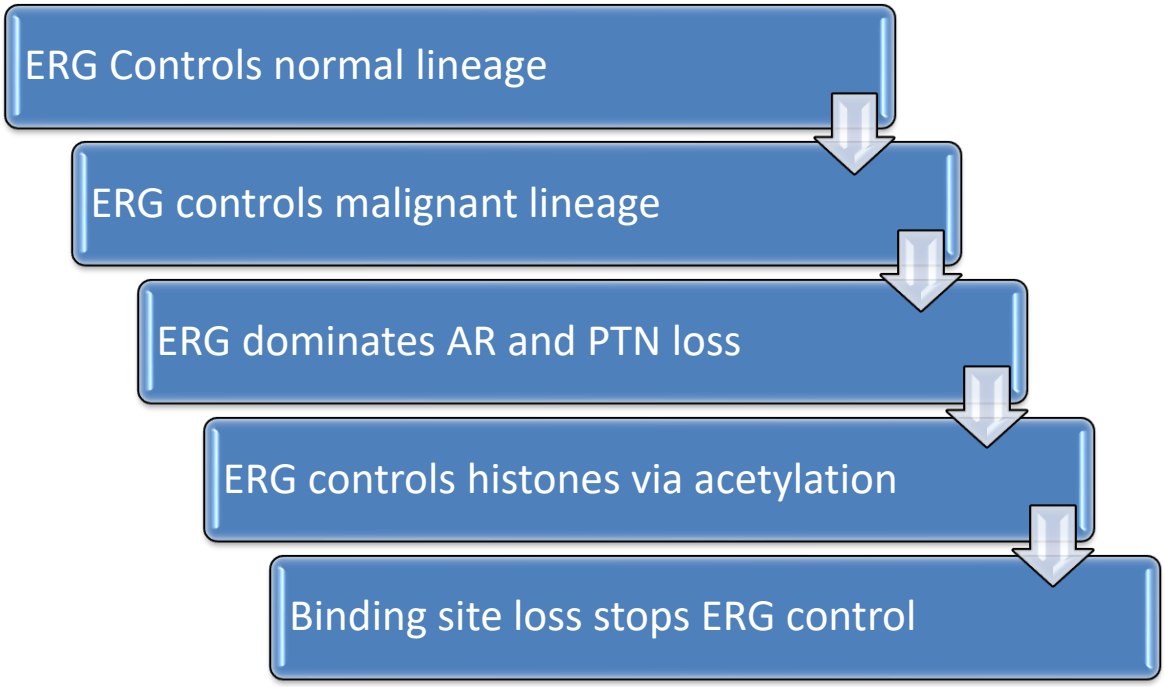


Thus, ERG can be seen in a more expanded light[3]. Not just as a fusion product but also having other significant properties. Also, this paper also raises the issues of the luminal versus basal cell as source of origin[4]. In affect Li et al argue that ERG is some type of master regulator. This argument based on in vitro studies and in vivo studies adds a significant dimension to the understanding of PCa.

As noted, Li et al talk of identifying a "master transcription factor". This concept has been debated over time but it does have merit. Namely that there exists a hierarchy in transcription factors and if one could identify the master one may have a target for a therapeutic. The logic appears to be as follows:

[3] https://www.researchgate.net/publication/315374473_ERG_TMPRSS2_AND_PROSTATE_CANCER

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

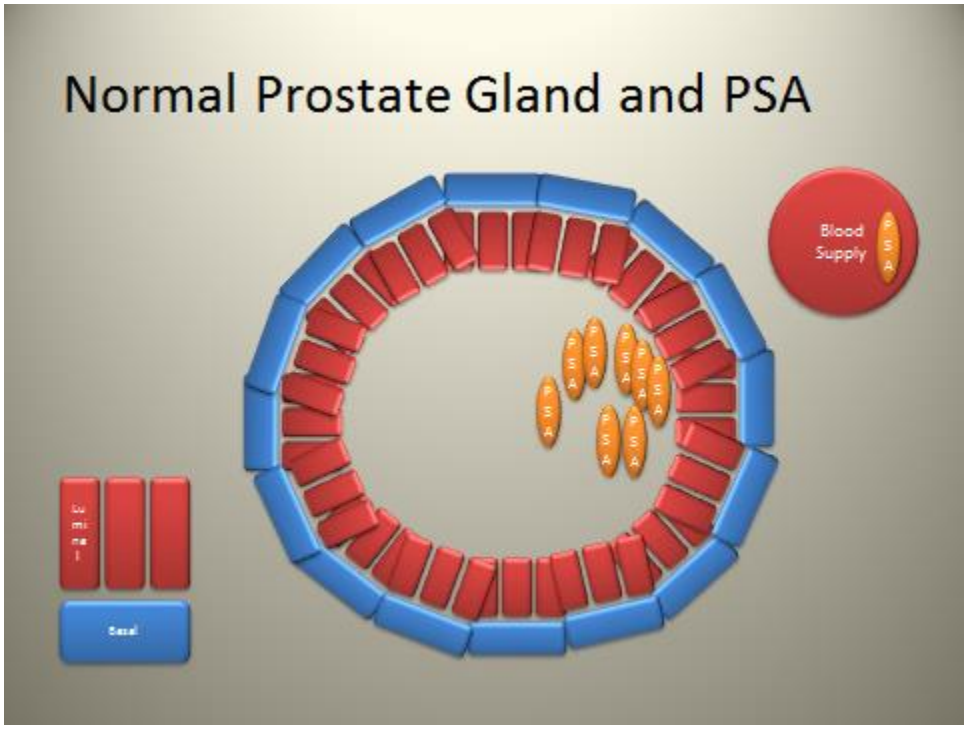

ERG Controls normal lineage
ERG controls malignant lineage
ERG dominates AR and PTN loss
ERG controls histones via acetylation
Binding site loss stops ERG control

2 LUMINAL VS BASAL

The prostate is basically a glandular body with a multiplicity of secreting regions. The regions have walls composed of basal cells and the internal lining is a layer of luminal cells.

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

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



PSA, prostate specific antigen, is a gene product of chromosome 19[5]. The PSA gene is androgen regulated. PSA is synthesized in the epithelial cells. It is secreted into the lumen of the prostate gland ducts and works its way into the serum most likely by diffusion. PSA tends to increase with hypertrophy and PCa. This most likely is due to cell proliferation and thus a larger base of excretion of PSA into the lumen. There does not however seem to be any studies relating serum PSA to prostate size, volume. A normal prostate is about 40 cc in volume and large prostates say of 100 cc may have more epithelial cells and thus putatively a larger PSA in the serum, however there does not appear to be evidence supporting this conjecture.

[5] See Kantoff, Prostate, p 213.

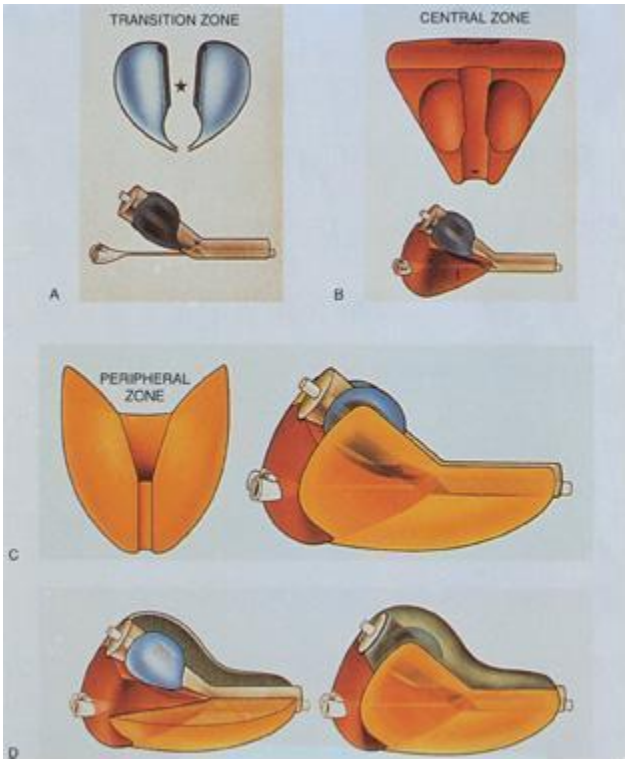
Most serum PSA is bound to proteins and there is some free. Thus the Percent Free PSA is often also measured. PSA released from cancer cells however is often not processed by intracellular proteolytic chains and thus is not free. High percent free is often a sign of no malignancy[6].

PSA velocity is another measure of malignancy potential. The definition of PSA velocity is the three sample average of PSA change per year or percent change per year. That is we take three time samples, and then calculate two velocities, from the second less first, and the third less second, and annualize each and take the average. If the velocity exceed 0.75 we have a threshold which requires examination[7].

2.1 THE NORMAL PROSTATE

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

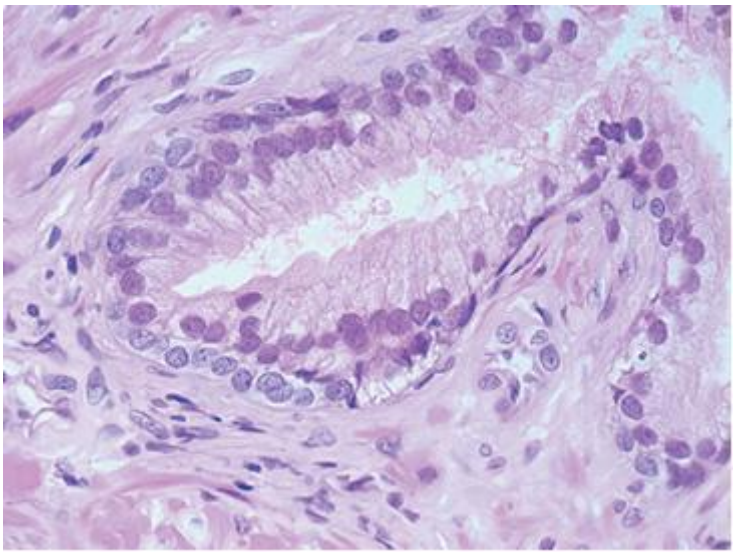
The basic structure of the prostate is shown below. It consists of three major zones; peripheral (dominant zone), central zone which is around the urethra), and the transition zone.



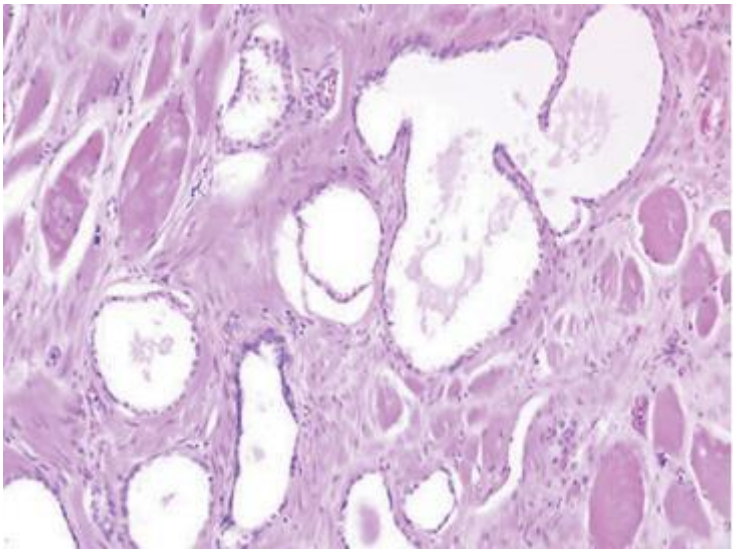
The cellular structure is depicted below. There are approximately 35-50 glands in the prostate, mostly in the peripheral zone and the glands have a lumen in which the prostatic secretions flow, and the glands have basal cells and luminal cells as shown below. The basal cells are dark and the luminal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

[6] Su, Prostate, p 5.
[7] Su, prostate. p 5.

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



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

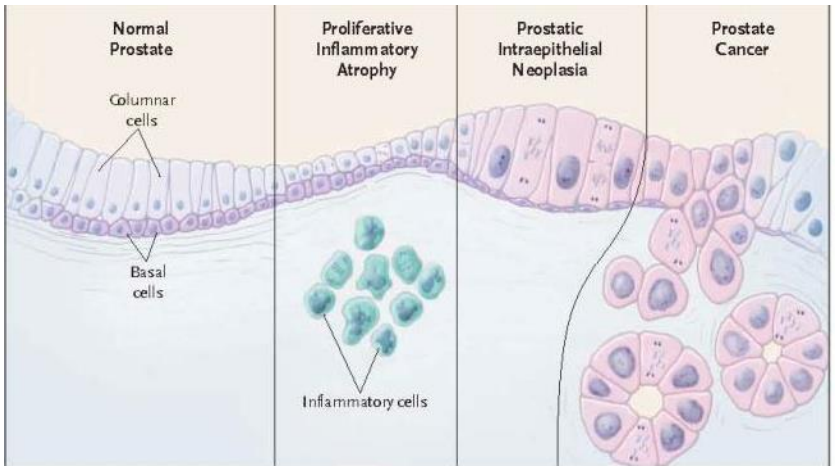


The normal prostate then is merely a collection of glands, glands composed of basal and luminal cells, with open glandular portions, the white areas above. As we noted before these glands emit various proteins and are an integral part of the male reproductive system.

2.2 SUMMARY OF PROSTATE STATES

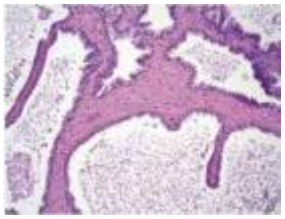
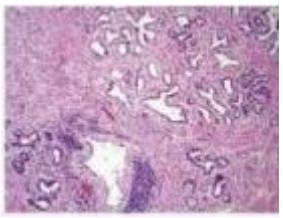
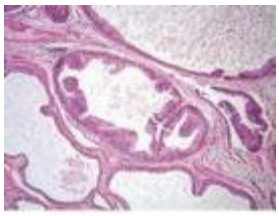
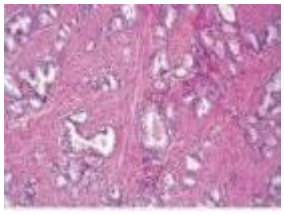
We now provide a high level summary of the changes in the prostate histologically as PCa is developed. We do this to lay out the various changes we will examine and to better understand what we may be looking for when developing pathways. We believe that it is essential that we always go back and forth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

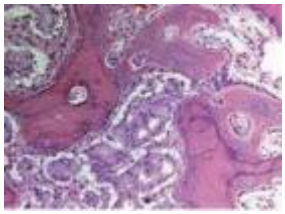
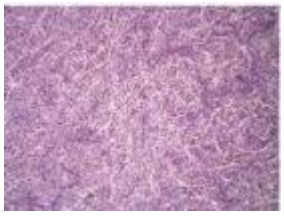
There is a general agreement, with of course many exceptions, as to the progression of prostate pathology and its related causes. A graphic from a recent NEJM article is shown below:



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

An excellent tabular summary from Taichman et al follows:

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

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

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

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

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

We shall now examine each of these in some detail.

2.3 PROSTATIC INTRAEPITHELIAL NEOPLASIA

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

First, we look at the histological structure of PIN and PCa. Cell changes occur and the changes morphologically are dependent upon the expression of or lack thereof of certain genes. The linking of morphology and gene expressions seems to fall short at this stage. Thus the nexus is missing.

Second, we look at some simple models for the development of HGPIN. As we have stated, the reason for this is twofold. First HGPIN is often assumed to be a natural precursor of PCa and as such one can assume that genetic changes necessary for PCa are first seen in HGPIN. Second we

know that HGPIN can suddenly regress and the cells revert to benign state. If that is the case and indeed it is one may ask if the genetic changes were the cause also of the regression or was there some exogenous cause. We focus primarily on the Goldstein et al model because it demonstrates both HGPIN and PCa and the relationship to morphological and genetic changes.

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

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

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

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

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

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

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

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

2.3.1 HGPIN Characterization

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

From the paper by Putzi and DeMarzo we have:

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

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

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

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

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

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

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

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

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

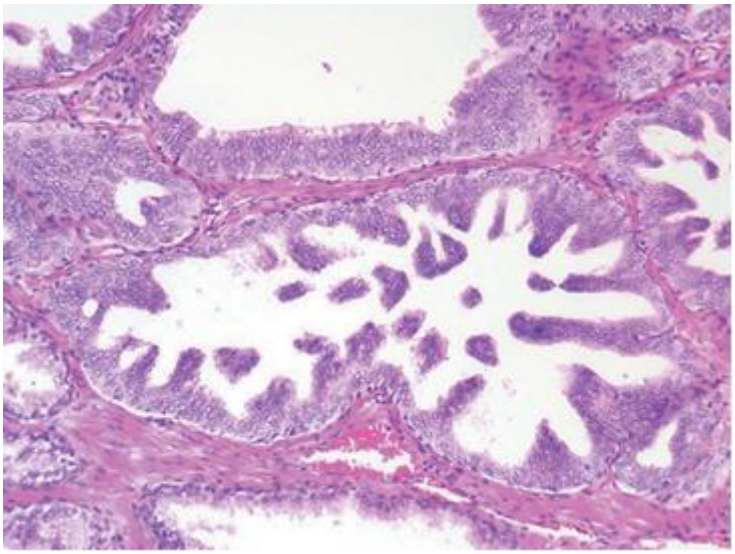
African-American men, who are at higher risk of prostate cancer mortality, appear to have a greater extent of PIN at any given age. PIN and prostate cancer lesions share a number of somatic genome abnormalities, including loss of DNA sequences at 8p and increased GSTP1 CpG island DNA methylation, among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

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

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

2.3.2 PIN Morphology

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



The PIN shows papillae which are shooting out within the gland and there is also significant basophillic staining of the papilla cells whereas the normal gland has limited staining of the luminal cells.

The key question is one of whether PIN is a precursor to PCa. Many articles state that it is but when one looks at the data there is still a significant area of doubt.

2.3.3 Some HGPIN Models

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

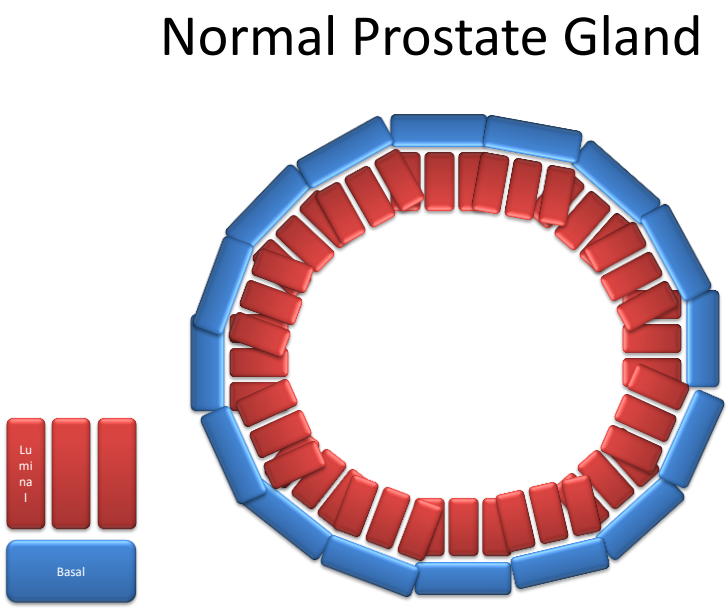
2.3.4 The Goldstein Model

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

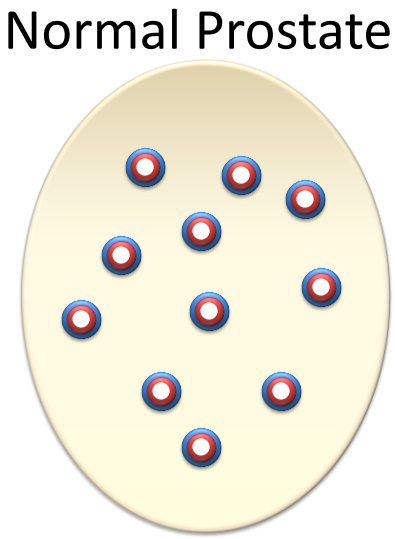
Simply what they did was to take two types of prostate cells, the basal and the luminal, tag them with surface tags, inject them into a mouse, and saw that only the basal cells grew, then they added two genes encoding for putative cancer pathways, and they saw that the basal cells grew to basal and luminal,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

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

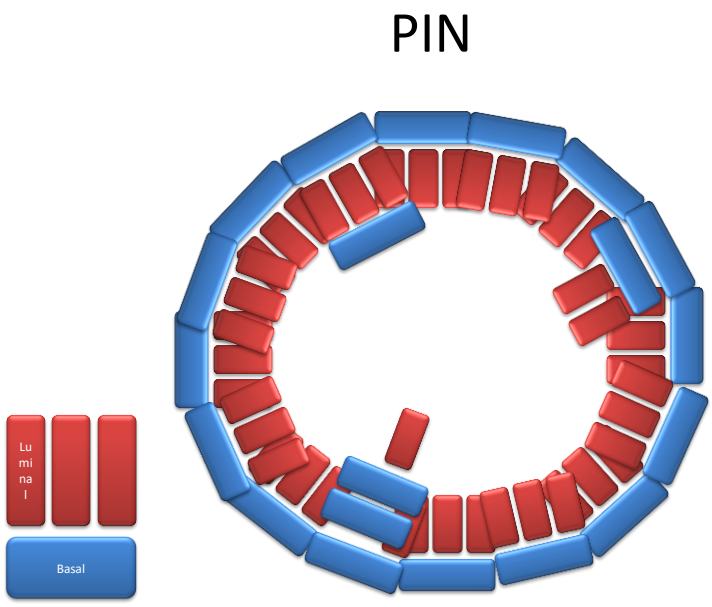
1. First the prostate has cell collections which act as glands with basal cells at the base and luminal cells on top. The luminal cells secret to the gland, the luminal space. This we show below.



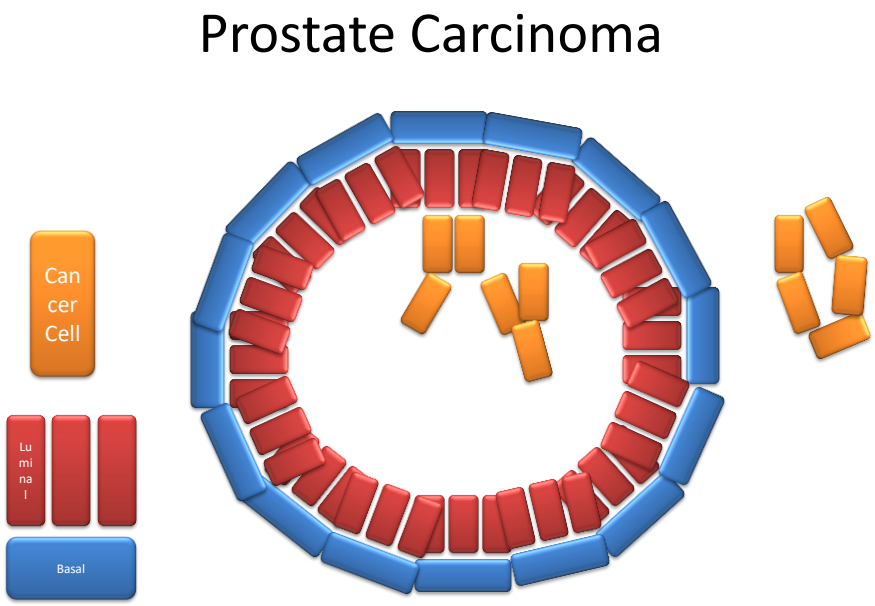
2. The normal prostate looks like what we show below, about 35-50 of these glands, and then surrounding material of muscle, blood supply, nerves, and lymphatics. The glands stand apart and they secret fluids into the lumen, the open parts of the gland. In between is the stroma composed of nerves, blood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



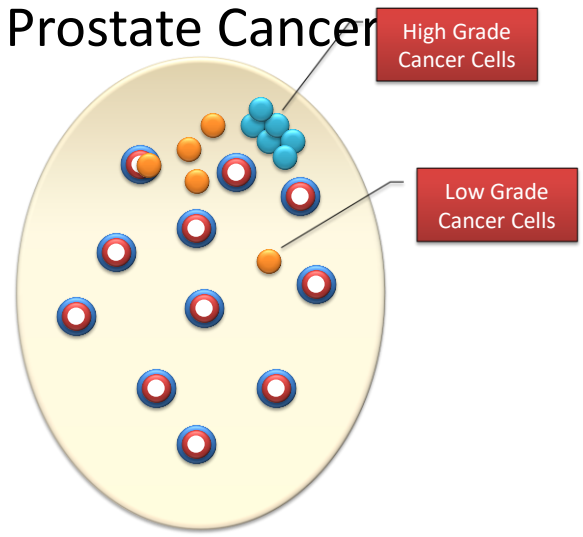
3. Now sometimes we see PIN, prostatic intraepithelial neoplasia, which is a growth of normal cells but not where they are to be. We may see the basal cells growing outwards and even some more luminal cells as well. The sign may be an increase in PSA since we have more luminal cells but the percent free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



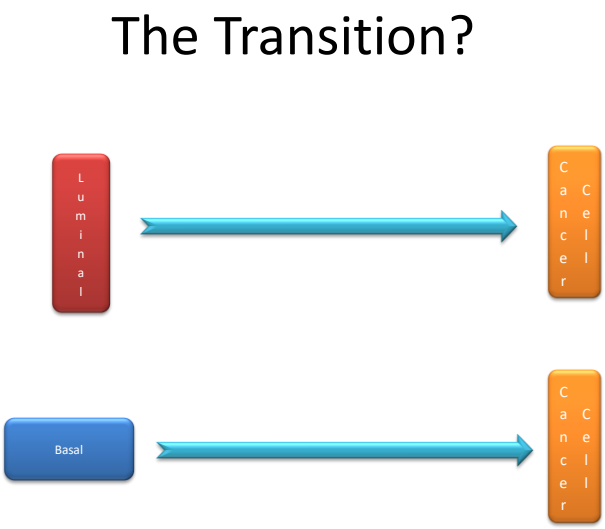
4. Then we may get prostate cancer, PCa, where the luminal cells types start to appear and grow without bound. The question is, where did these cells come from, other luminal cells or basal cells, or what. This is the question that the authors addressed with this elegant experiment. There is also the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



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



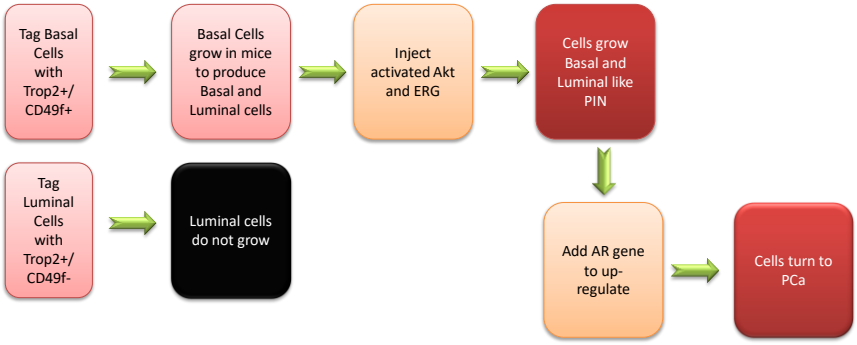
5. Thus the question posed by the authors was the one which asks from what cell does cancer begin? Their answer suggested by the Goldstein et al model is the basal cell. We will see that there is great controversy in this answer and furthermore the Li model contradicts it.



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

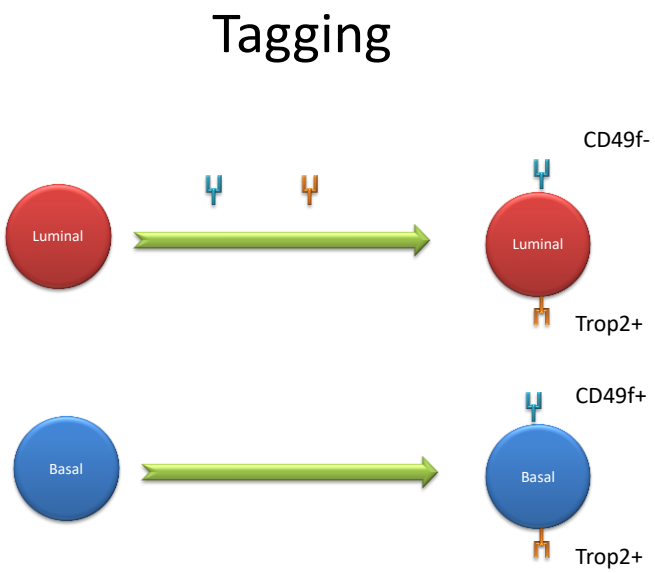
But the authors took a simple approach and looked at three genes in the putative pathway process. This is shown below:

Goldstein Process



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

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



As to the two initial genes we have:

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

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

This study still leaves several open questions:

1. Is the clonal theory of cancer still standing or can a single cell transform and then induce other cells via chemical signaling.

2. Is the basal cell the only one. There appears to be some issues here and the review article looks at these.

3. Is PIN an artifact or a precursor. Clinically men with PIN have a slightly higher risk of PCa but not a substantially higher as would be argued in this model. In fact men with PCa do not always have PIN and men with PIN do not always get PCa.

4. Is this just an artifact pathway, the true pathway, one of many pathways.

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

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

2.3.5 Other Models

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

1. Yen et al (2003) have reported on a murine model which demonstrated that by implanting c-Myc genes into a mouse that it resulted in murine PIN and then shortly thereafter PCa. Yen et al also shown loss of NKX3.1, a tumor suppressor gene, which is putatively involved in PCa as well as PIN. NKX3.1 is a 8p21 gene whose function is to generate the Homeobox protein[8]. It is known to be suppressed in familiar prostate cancer and in the case of Yen it is reduced in its expression as well.

2. Lawton and Witte discuss the generation of PIN by means of lentivirus infection via an siRNA which is a knock out for PTEN.

2.3.6 HGPIN, A Precursor of PCa?

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

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

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

2.3.6.1 Key Questions

Let us begin with what we assume is known:

[8] Pecorino, Cancer, p 114.

1. HGPIN is driven by underlying progressive and non-changeable changes in the genetic structure of benign cells in the prostate glands.

2. There is a putative association between HGPIN and PCa, reflected in an increased incidence of PCa when HGPIN is present.

3. PCa like most other cancers is characterized by the clonal model, namely one cell becomes aberrant and all other cancers cells are daughter cells of the aberrant clone.

4. PCa is known to result via a set of genetic changes resulting in the cell growth outside of the gland and the creation of malignant glandular structures wherein additional genetic changes occur and result in a less structured morphology and then metastasis.

5. HGPIN regression is seen. This means that the HGPIN cells totally disappear resulting in a purely benign appearance of the prostate glands. It begs the question of; do they cells die or are they attacked and destroyed or is there some reversion mechanism? PIN is a proliferation, so any continuation of cell existence would imply at best a morphological change of say the nucleus and nucleoli but not the total cell count, namely the clustering of many cells in the gland. Thus in regression we do not know what happens or how.

Thus these observations pose the following questions:

1. What causes the disappearance of multiple clusters of HGPIN? Is it apoptosis of some form, an immune response, a genetic switch, or something else?

2. Has there been any extensive studies of HGPIN regression to understand how it arises?

3. If HGPIN regression is based upon some to-be-understood mechanism, can that same mechanism be applied in some form to PCa?

4. Does HGPIN, which is regressionable, have certain cell surface markers which are presentable to the immune system and thus enable enhanced immune responses.

5. Is there a stem cell created when PCa evolves and is PIN lacking in such a stem cell?

The literature demonstrates how to create PIN. There are a few presentations on how to regress PIN[9]. However the nexus of forward PIN progression and backward PIN regression is not complete. We attempt herein to review this in some detail and then to place it in a structure for further analysis and study.

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

[9] Narayanan et al using NSAID.

2.3.6.2 An Example

Let us begin with a simple example. A 68 year old male is examined due to an increase in PSA from 1.5 to 2.3 in a one year period. The DRE is normal but there is a family history of a first degree relative who died from an aggressive PCa, at 78 years of age. Re-measuring the PSA from two independent sources yields values of 1.8 and 1.9 two months after the raised PSA. A 14 core biopsy is performed and the results are as follows:

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

After an eight month period PSA was measured again and this time it was 2.0. A second biopsy was performed using 16 cores. The results are:

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

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

2.3.7 PCa Histology and Grading

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

2.3.8 Prostate Cancer Histology

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

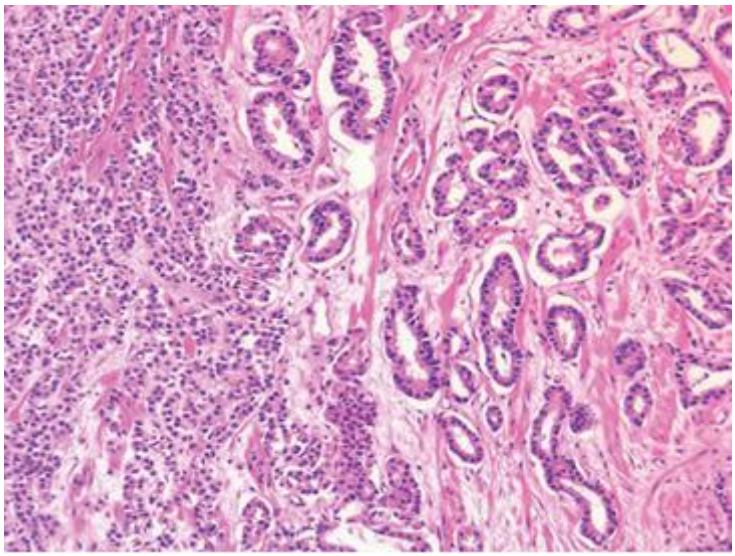
We will use the Gleason grading score as a means to characterize the level of cancer progression within the prostate.

2.3.9 Grading

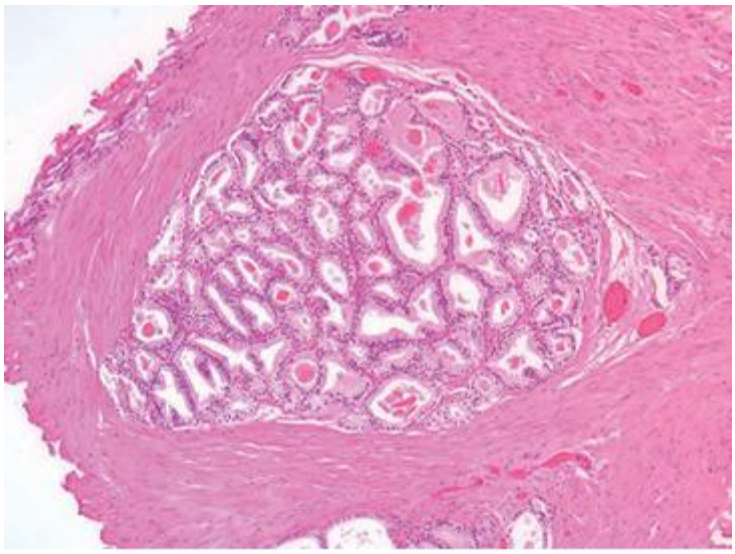
We present the grading system developed by Gleason. On the one hand this has been used as a gold standard for ascertaining future progress and yet it is still just a morphological tool. It fails to determine the pathways and regulators in a cell by cell basis.

1.1.1.1 Gleason 1

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



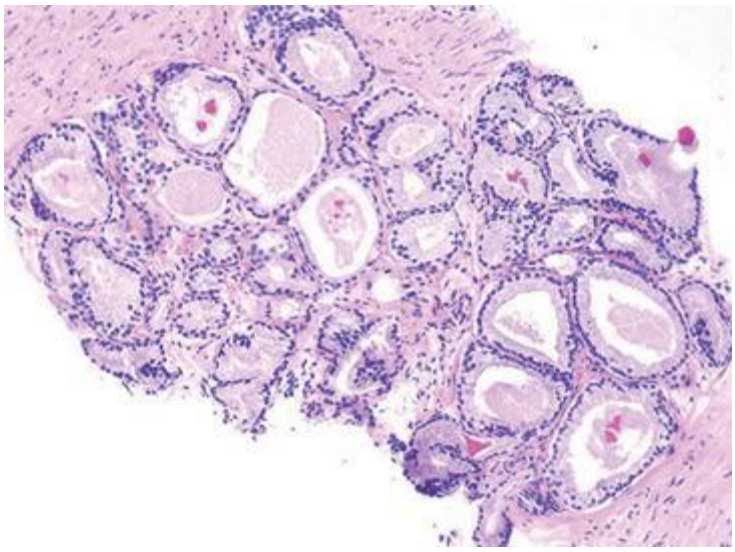
We show another view of a Gleason 1 below. This is especially descriptive of such a form because it appears almost as a single and isolated structure. The interesting question will be if this is PCa then if PCa is clonal is this cluster an aberrant outgrowth of a normal cells, if so which one, and if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



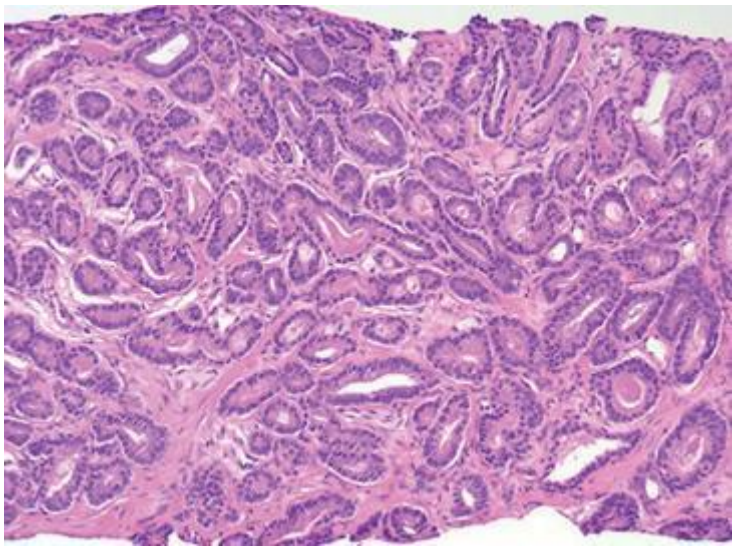
2.3.9.1 Gleason 2 and 3

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

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

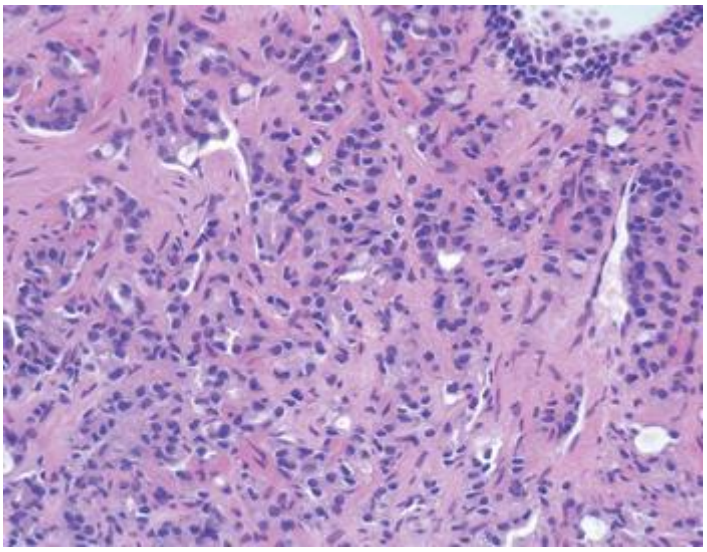


A Gleason 3 throughout is shown below.

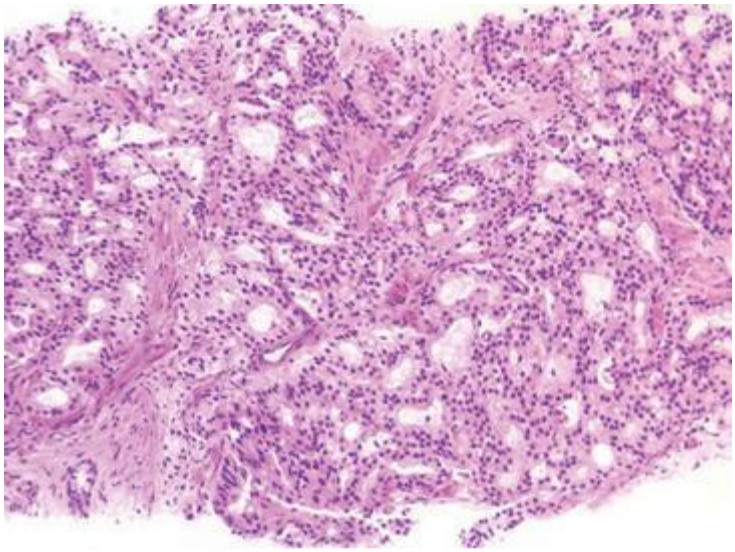


2.3.9.2 Gleason 4

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

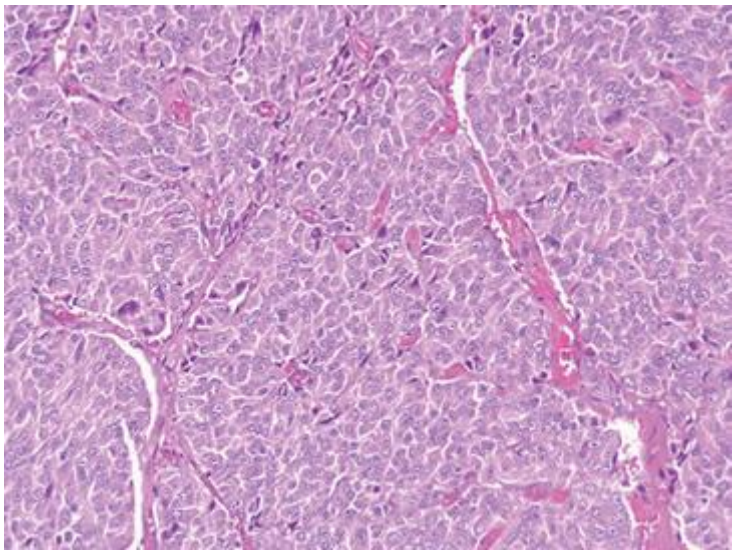


A Gleason all 4 is shown below. Note that the cells are sticking closed and the entire mass appears as a sieve like mass.



2.3.9.3 Gleason 5

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



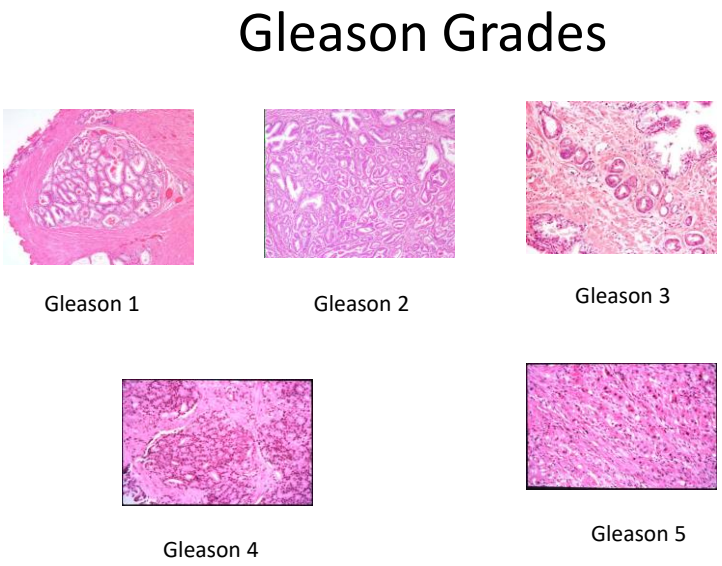
2.3.10 Gleason Summary

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

We repeat the grading commentary below.

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

The following chart is a summary of the progression.



2.3.11 Models From Grading

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

1. Movement from existing benign gland to a separate but glandular like proliferation. Cells which would normally remain dormant go through a replication cycle, apoptosis and cell proliferation control seems lost. New glands appear clustered but appear separate.

2. Growth of the new glands makes them expand but remain morphologically glandular. They close packing begins to disappear and glands start to stand on their own. It is as if they are expanding and growing and the basal layer begins to disappear. Luminal like cancer cells start to be predominant.

3. Many small micro-glands start expanding and cell growth accelerates and the cells appear more cancer like but there is still some morphological glandular structure left.

4. The many glands have dramatically different shaped and start closing in one another and appear sieve like with small openings. They look as if they are losing any intercellular communications resulting is a common mat of cells.

5. Cells have lost any morphological form related to glands and appear as a mat of cancer cells replacing the stroma totally. No intercellular communications is left and cellular growth control has been eliminated totally.

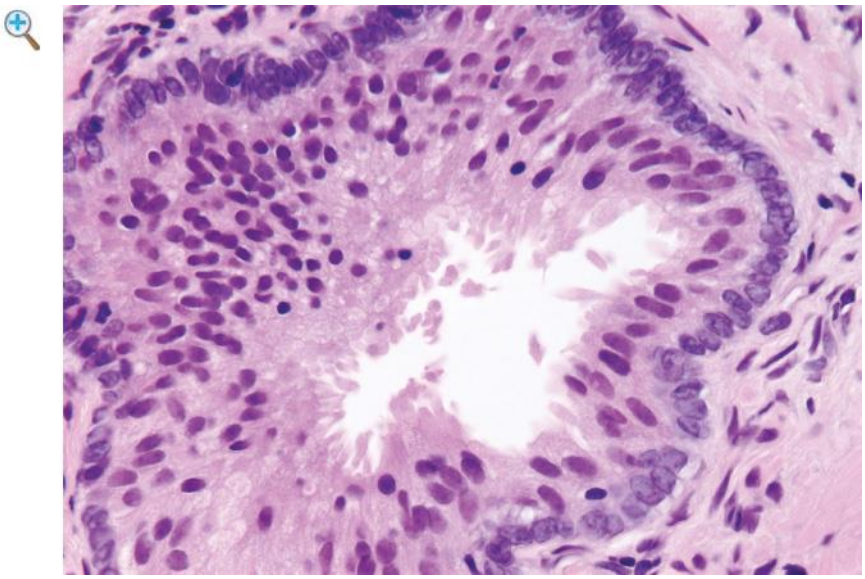
These five steps are consistent with the Gleason grading but they also parallel the way the intracellular and intercellular controls are lost. We will look at these mechanism later.

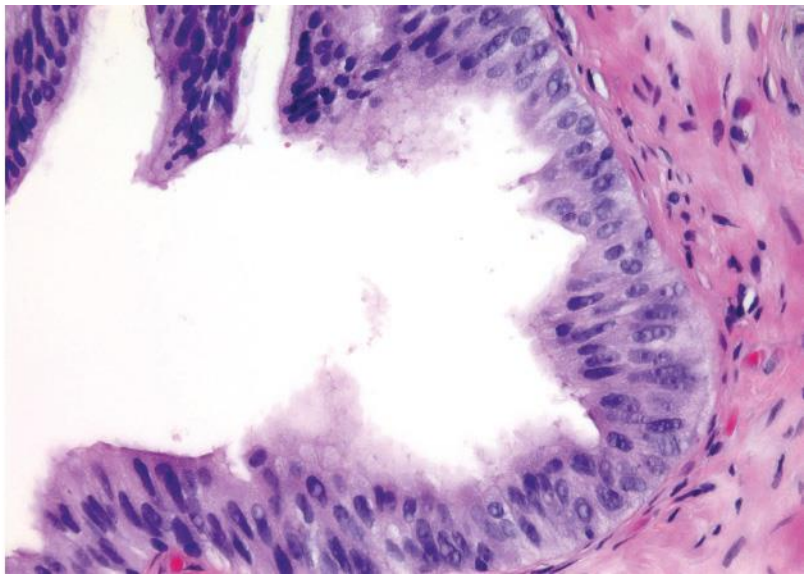
2.4 HGPIN AND PCA

From Epstein we have the following discussion:

Similar to prostatic adenocarcinoma, HGPIN can show 8p12-21 allelic loss and gain of chromosomes 7, 8, 10, and 12.17–19About 20% of HGPIN lesions have a TMPRSS2-ERG fusion gene, which is a common molecular abnormality detectable in about 50% of prostate cancers.20,21 Next-generation sequencing and in silico analysis of evolutionary ages predicted that HGPIN genomes were much younger than prostatic adenocarcinoma genomes. Their data supported that HGPIN is the direct antecedent of prostatic adenocarcinoma, typically requiring additional genomic alterations to progress to invasive carcinoma.22,23 All these findings would be expected if HGPIN is a precursor lesion to carcinoma of the prostate.

It has been shown that HGPIN is more closely related to peripheral, as opposed to transition zone cancers. This weaker association of HGPIN to low-grade transition zone carcinomas is also supported by the histologic differences of HGPIN and transition zone carcinomas.24 Centrally located low-grade adenocarcinomas tend to have bland cytology, often lacking nuclear enlargement or nucleoli in contrast to HGPIN. Peripherally located intermediate-grade carcinomas often have identical cytologic features to those of HGPIN. However, not all prostate cancers arise from HGPIN. The majority of prostates with early carcinomas lack any HGPIN within the entirely embedded prostate glands. In addition, even in prostate glands where there exists both early cancer and HGPIN, in only one-third of the cases is the HGPIN adjacent to the cancer





3 ERG

The ETS transcription family contains many elements and ERG is one of them. As Hollenhorst et al note:

ETS proteins are a group of evolutionarily related, DNA-binding transcriptional factors. These proteins direct gene expression in diverse normal and disease states by binding to specific promoters and enhancers and facilitating assembly of other components of the transcriptional machinery. The highly conserved DNA-binding ETS domain defines the family and is responsible for specific recognition of a common sequence motif, 5-GGA(A/T)-3.

Attaining specificity for biological regulation in such a family is thus a conundrum. We present the current knowledge of routes to functional diversity and DNA binding specificity, including divergent properties of the conserved ETS and PNT domains, the involvement of flanking structured and unstructured regions appended to these dynamic domains, posttranslational modifications, and protein partnerships with other DNA-binding proteins and . The review emphasizes recent advances from biochemical and biophysical approaches, as well as insights from genomic studies that detect ETS factor occupancy in living cells.

The authors continue:

Rearrangements of ETS loci are a hallmark of prostate cancer with ~50% of tumors showing alterations at an ETS gene locus. In the most common scenario, a promoter region from an androgen-responsive, prostate-specific gene, TMPRSS2, is attached to the ERG locus to drive aberrantly regulated ERG expression in prostate cells. Chromosomal rearrangements that result in the overexpression of members of the PEA3 subfamily (ETV1, ETV4, or ETV5) are found less frequently. These genetic changes are implicated in tumorigenesis. … The involvement of the AR in normal prostate development, coupled with the prevalence of ETS-binding sequences at AR-bound regions, implies a cooperative interaction that could play a role in ETS mediated prostate oncogenesis.

Expression studies indicate that a potential mechanism of oncogenic ETS proteins, such as ERG and ETV5, at these sites is antagonism of normal AR function. Supporting this idea, tumor-suppressor ETS proteins, such as SPDEF, synergize with the AR. However, the genetic finding that ETS and AR genes cooperate in oncogenesis indicates that this mechanism is more complex than our current understanding and may differ on a target-by-target basis. The biochemical mechanism for interactions between ETS factors and the AR is also not known.

There is some evidence that the AR can physically interact with ERG (139), ETV5 (179), SPDEF (66), ETV1 (181), and ETS1 through their ETS domains, even in the absence of DNA. However, whether this interaction has any ETS family specificity or how it impacts ETS function remains unknown. DNA-binding assays have not tested cooperative binding, and bioinformatics analyses have not identified an overrepresented spacing of ETS- and AR-binding sites functionally analogous to that observed with the ETS1/RUNX partnership. Therefore, the interaction between ETS proteins and the AR may lead to specificity of function, but not necessarily cooperative DNA

binding. Thus, ETS proteins may influence cancer via AR target genes, but the mechanism of this effect remains to be discovered

As Mosquera et al note:

Fusion of theTMPRSS2 prostate-specific gene with the ERG transcription factor is a putatively oncogenic gene rearrangement that is commonly found in prostate cancer tissue from men undergoing prostatectomy.

However, the prevalence of the fusion was less common in samples of transurethral resection of the prostate from a Swedish cohort of patients with incidental prostate cancer followed by watchful waiting, raising the question as to whether the high prevalence in prostatectomy specimens reflects selection bias.

We sought to determine the prevalence of TMPRSS2-ERG gene fusion among prostate-specific antigen screened men undergoing prostate biopsy in the United States ..

Our results show that this gene rearrangement is common among North American men who have prostate cancer on biopsy, is absent in benign prostate biopsy, and is associated with specific morphologic features. These findings indicate a need for prospective studies to evaluate the relationship of TMPRSS2-ERG rearrangement with clinical course of screening-detected prostate cancer in North American men, and a need for the development of noninvasive screening tests to detect TMPRSS2-ERG rearrangement.

As NCBI notes[10]:

This gene encodes a member of the erythroblast transformation-specific (ETS) family of transcriptions factors. All members of this family are key regulators of embryonic development, cell proliferation, differentiation, angiogenesis, inflammation, and apoptosis. The protein encoded by this gene is mainly expressed in the nucleus. It contains an ETS DNA-binding domain and a PNT (pointed) domain which is implicated in the self-association of chimeric oncoproteins. This protein is required for platelet adhesion to the subendothelium, inducing vascular cell remodeling. It also regulates hematopoesis, and the differentiation and maturation of megakaryocytic cells.

This gene is involved in chromosomal translocations, resulting in different fusion gene products, such as TMPSSR2-ERG and NDRG1-ERG in prostate cancer,

EWS-ERG in Ewing's sarcoma and FUS-ERG in acute myeloid leukemia. More than two dozens of transcript variants generated from combinatorial usage of three alternative promoters and multiple alternative splicing events have been reported, but the full-length nature of many of these variants has not been determined.

This is officially known as "ETS transcription factor ERG".

[10] https://www.ncbi.nlm.nih.gov/gene/2078

As Perner et al have noted earlier:

Prostate cancer is a common and clinically heterogeneous disease with marked variability in progression. The recent identification of gene fusions of the 5-untranslated region (UTR) of TMPRSS2 (21q22.3) with the ETS transcription factor family members, either ERG (21q22.2), ETV1 (7p21.2; ref. 1), or ETV4 (17q21; ref. 2), provides a mechanism for overexpression of ETS genes in prostate cancer. TMPRSS2 is highly expressed in prostate cancer and contains androgen response elements in the promoter. Recent work showed that exposure to androgen regulates the fused ETS family member. We observed that in the TMPRSS2:ERG positive prostate cancer cell line VCap exposure to a synthetic androgen specifically increased ERG expression, whereas no change in expression was observed in the TMPRSS2:ERG-negative LNCaP prostate cancer cell line

As Krumbholz et al have recently noted:

There is increasing interest in the use of cell-free circulating tumor DNA (ctDNA) as a serum marker for therapy assessment in prostate cancer patients. Prostate cancer is characterized by relatively low numbers of mutations, and, in contrast to many other common epithelial cancers, commercially available single nucleotide mutation assays for quantification of ctDNA are insufficient for therapy assessment in this disease.

However, prostate cancer shares some similarity with translocation-affected mesenchymal tumors (e.g., leukemia and Ewing sarcoma), which are common in pediatric oncology, where chromosomal translocations are used as biomarkers for quantification of the tumor burden.

Approximately 50% of prostate cancers carry a chromosomal translocation resulting in generation of the TMPRSS2-ERG fusion gene, which is unique to the tumor cells of each individual patient because of variability in the fusion breakpoint sites.

In the present study, we examined the structural preconditions for TMPRSS2-ERG fusion sites in comparison with mesenchymal tumors in pediatric patients to determine whether the sequence composition is suitable for the establishment of tumor-specific quantification assays in prostate cancer patients. Genomic repeat elements represent potential obstacles to establishment of quantification assays, and we found similar proportions of repeat elements at fusion sites in prostate cancer to those reported for mesenchymal tumors, where genomic fusion sequences are established as biomarkers.

Our data support the development of the TMPRSS2-ERG fusion gene as a noninvasive tumor marker for therapy assessment, risk stratification, and relapse detection to improve personalized therapy strategies for patients with prostate cancer.

3.1 TPMRSS2-ERG

One of the few known fusions or translocations involved in PCa is the TMPRSS2 and ERG fusion. It is seen in PCa as well as HGPIN. Its presence is known to be a marker for highly aggressive PCa.

3.2 ETS FAMILY

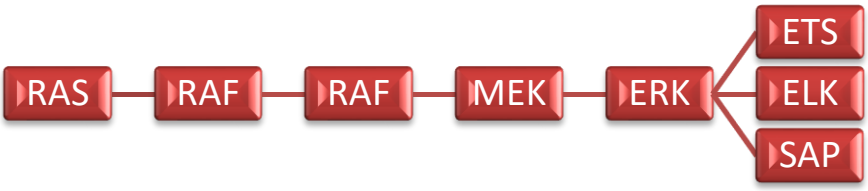
The ETS family of genes are positive or negative regulators of gene expression. They can up or down regulate expression. They are named for the initial gene discovered, the E26 Transforming Sequence, where E26 was the oncogene characterized in 1986 of an avian transforming virus called E26. It is also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

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

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

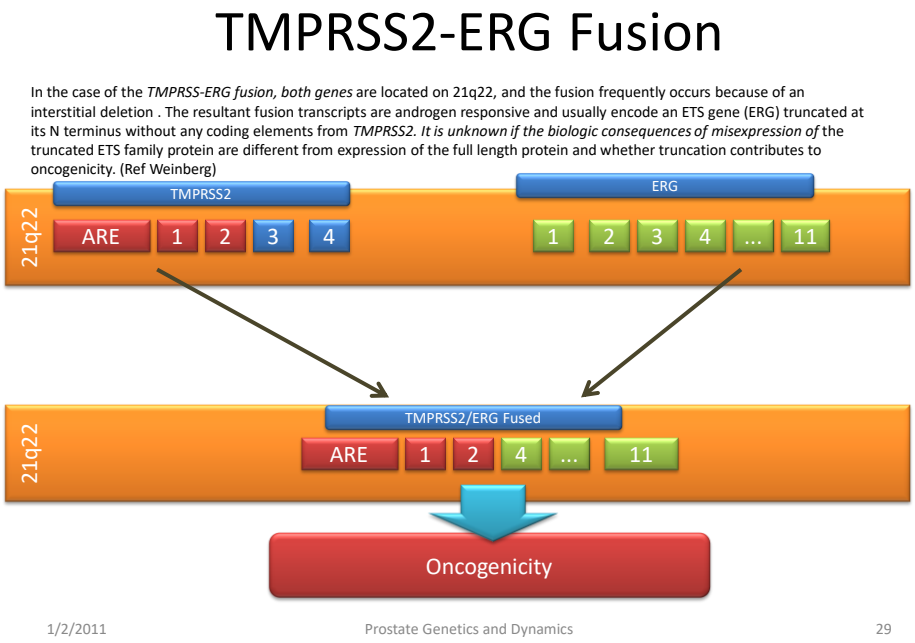
The ERG gene was first presented in the paper by Reddy et al in 1987. There the authors identified it and set it in the ETS family.

From Weinberg, we see that the ETS are transcription factors driven by the RAS/RAF pathway along with other such factors.

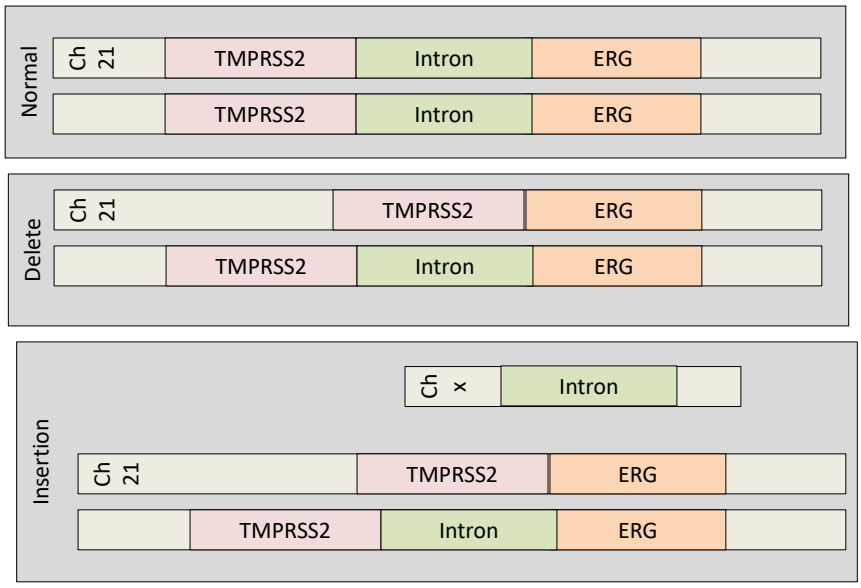


3.3 TMPRSS2 AND ERG FUSION

The fusion of TMPRSS and ERG is another genetic promoter of PCa and it is the primary translocation gene seen.



Tomlins et all discuss the various conjectures regarding the fusions. The graphic below is based upon Tomlins et al. The example below shows the normal state on 21 and then the deletion, the intron is just removed, and then an insertion where the intron is removed but inserted elsewhere. See also the work by Mani et al (2009) regarding the gene fusions in general as applied to PCa. Also the work by Demichelis et al (2009), Marucci et al (2007) Iljin et al (2006) and Esgueva et al 2010) for extensions of this description.



It is thus the fused gene that cause the problem acting as an oncogene. This is unlike the other processes, for here we actually have genetic changes in location. The intron is 3Mb long so it is a nontrivial deletion. Unlike a methylation of a base pair element this requires substantial genetic change.

As the work of King et al state:

These data suggest that TMPRSS2-ERG is insufficient to initiate prostate neoplasia and that cooperating oncogenic lesions are required. Two relatively common abnormalities in human prostate cancer are PTEN loss and MYC amplification, both of which have pathogenic roles in genetically engineered mouse models

In a 2005 paper by Tomlins et al the authors discuss the fusion of the two genes, TMPRSS2 and ERG and the prevalence of this fusion in PCa. They relate the translocation and fusion of the genes in CML where BCR-ABL is fused to create a new gene, with an associated translocation, and then discuss the juxtapositioning of promoter and enhancers of one gene being juxtaposed to a proto-oncogene. Using a technique calls Cancer Outlier Profile Analysis, COPA, they had managed to isolate the fused product of TMPRSS2 and ERG in PCa. This is a fusion on 21q22. See also the work by Rubin and Chinnaiyan (2006) on the COPA analysis.

In the work of Esgueva et al the authors indicate that this fusion has several distinct features:

1. Murine models with overexpressed ERG with and without PTEN loss show a neoplastic phenotype.

2. ERG and histological features have been correlated. This is detailed in the paper by Mosquera et al (2007).

3. Specific pathways have been shown to have been rearranged, especially estrogen signalling.

4. Somatic copy number alterations have been found to be increased in ERG enhance PCa.

5. ERG rearranged PCa have highly negative outcomes.

6. ERG rearranged response to abiraterone is different.

The conclusion that Esgueva et al then reach is that ERG rearrange PCa is a different clinical class.

3.4 TMPRSS2:ERG HGPIN

There have been several studies on the relationship of this fusion to HGPIN. In Mosquera et al they state:

Given the more aggressive nature of TMPRSS2-ERG prostate cancer, the findings of this study raise the possibility that gene fusion-positive HGPIN lesions are harbingers of more aggressive disease. To date, pathologic, molecular, and clinical variables do not help stratify which men with HGPIN are at increased risk for a cancer diagnosis. Our results suggest that the detection of isolated TMPRSS2-ERG fusion HGPIN would improve the positive predictive value of finding TMPRSS2-ERG fusion prostate cancer in subsequent biopsies.

The authors then continue regarding HGPIN:

In the United States, approximately 1,300,000 prostate biopsies were done in 2006 with the detection of 234,460 new cases of prostate cancer. The incidence of isolated high-grade prostatic intraepithelial neoplasia (HGPIN) without carcinoma ranges from <1% to 16% , and the risk of finding carcinoma on subsequent biopsies is 10% to 39% [median risk of 24% (6)] depending on the time of repeat biopsy and number of cores.

A decline in the predictive value of HGPIN for prostate cancer to 20% in contemporary needle biopsies is most likely due to extended biopsy techniques that yield higher rates of cancer detection. Both HGPIN and prostate adenocarcinoma share molecular anomalies, including telomere shortening, RAR hypermethylation, allelic imbalances, and several chromosomal anomalies and c-myc amplification. Overexpression of p16, …, and altered proliferation and apoptosis in HGPIN and prostate cancer have also been shown…In particular, the TMPRSS2-ERG gene fusion prostate cancer is associated with higher tumor stage and tumor-specific death or metastasis . Two recent studies have shown the presence of TMPRSS2-ERG gene fusion in 20% of HGPIN lesions…

In a detailed study of murine models, Zong et al have concluded further the following:

1. *ERG Overexpression in Adult Murine Prostate Cells Results in Epithelial Hyperplasia and Focal PIN Lesions.*

2. *ERG-Transduced Prostate Glands Display a Skewed Cell Lineage Composition with Loss of Cytokeratin 5 (CK5)-Positive Basal Cells and Increased CD49f Expression in Luminal Cells.*

3. *ERG Overexpression Induces Up-Regulation of c-Myc and c-Jun Protein in Primary Prostate Epithelia.*

4. *Combined ERG Overexpression and p53 Deletion in Prostate Epithelia Does Not Result in Invasive Adenocarcinoma.*

5. *ERG Collaborates with Aberrant PI3K Pathway to Promote PCa Progression. Deletion of the tumor suppressor PTEN occurs in 68% of human PCas and results in activation of the PI3K pathway. We demonstrated that increased PI3K signaling via shRNA-mediated PTEN knockdown or overexpression of an activated form of AKT in murine prostate cells causes PIN lesions in the tissue-regeneration model. In this study, we combined overexpression of ERG and activated AKT and found that grafts derived from co-infected adult prostate cells weighed 2–3 times more than grafts generated from AKT or ERG overexpression alone. In contrast to AKT-induced PIN lesions, the prostate glands that simultaneously overexpressed ERG and AKT/GFP exhibited a cribriform growth pattern with cell crowding and embedded acini. The cells in these proliferative foci exhibited nuclear atypia, evidenced by hyperchromatic nuclei, mitotic figures, nuclear contour irregularity, and enlargement. These findings suggest that high levels of ERG protein collaborate with constitutively activated AKT kinase, leading to the development of invasive PCa.*

6. *High Levels of ERG Fully Transform Primary Prostate Cells Through Synergy with Enhanced AR Signaling. AR is commonly mutated or amplified in human PCa, and the AR pathway is the most extensively studied pathway in PCa because of its role in late-stage hormone-refractory PCa. Given that up-regulation of ETS transcription factors is mainly driven by the androgenresponsive TMPRSS2 promoter in most samples of human PCa, it is reasonable to hypothesize that both ETS overexpression and AR signaling coexist in malignant prostate epithelial cells.*

As we have discussed before, the subsequent work by Goldstein et al took this a step further and in murine models demonstrated the development of PIN and then PCa. However, the murine model is not exactly projectable to the human. In addition, there is no viable reverse path from HGPIN to benign cells. In fact the work of Demichelis et al indicate that watchful waiting, the proverbial do nothing strategy, is somewhat effective except in TMPRSS2:ERG fusion cases. However, the determination of the gene fusions is currently not common in prostate biopsies.

3.5 TMPRSS2:ERG PATHWAYS AND CONTROL

There currently is limited pathway modeling of this fusion effect. We demonstrated the Weinberg ETS model and there is work by Yu et al showing AR control effects but no clear definitive pathway models seems to exist. A similar analysis of the AR driving of the ERG promoters is performed by Dobi et al (2010). Dobi et al conclude:

Expression of the ERG proto-oncogene, is activated in 50-70% of prostate tumors by androgen receptor (AR) mediated signals due to the fusion of AR regulated promoters (primarily TMPRSS2 and to a lesser extent SLC45A3 and NDRG1) to the ERG protein coding sequence.

Our previous studies of quantitative expression levels of ERG or TMPRSS2-ERG fusion transcripts have noted that relatively low or no ERG expression in prostate tumors significantly associated with progressive disease. Here, we have tested the hypothesis that ERG expression levels in prostate tumor cells reflect AR transcriptional regulatory function in a given biological context of the tumor progression.

Therefore, tumors with lower ERG may represent a subset with attenuated AR signaling. Expression of ERG and other AR regulated genes were evaluated …. Overall, ERG expression pattern was similar to that of other AR regulated genes. Strikingly low frequency of ERG expression was noted in PD tumor cells (30%) in comparison to WD tumor cells (80%), suggesting for subdued AR function in a significant fraction of tumors with genomic alterations of ERG. By integrating ERG into a panel of defined AR target genes, we developed a cumulative AR Function Index (ARFI), which if validated may have future potential in stratifying patients for targeted therapy on the basis of overall AR functional status in primary tumors….

Taken together, the ARFI approach reported here, if developed further has potential to stratify prostate tumors on the basis of in vivo functional status of AR which could lead to development of new paradigms in the treatment selection of patients for androgen ablation or other therapies. For example patients with ARFI positive versus ARFI negative/attenuated tumors may be identified in early stages of disease and latter may be more responsive to non-androgen ablation focused strategies.

Along similar lines patients with ERG gene fusion but not expressing ERG may not benefit from a potential ERG targeted therapy. Alternatively patients with varying degree of ARFI positivity may need different androgen ablation therapy strategies. Finally, association of low or no ERG in a large percentage of poorly differentiated tumors appears to be either reflection of attenuated AR signaling in tumors harboring ERG fusions or a distinct class of tumors without ERG alterations.

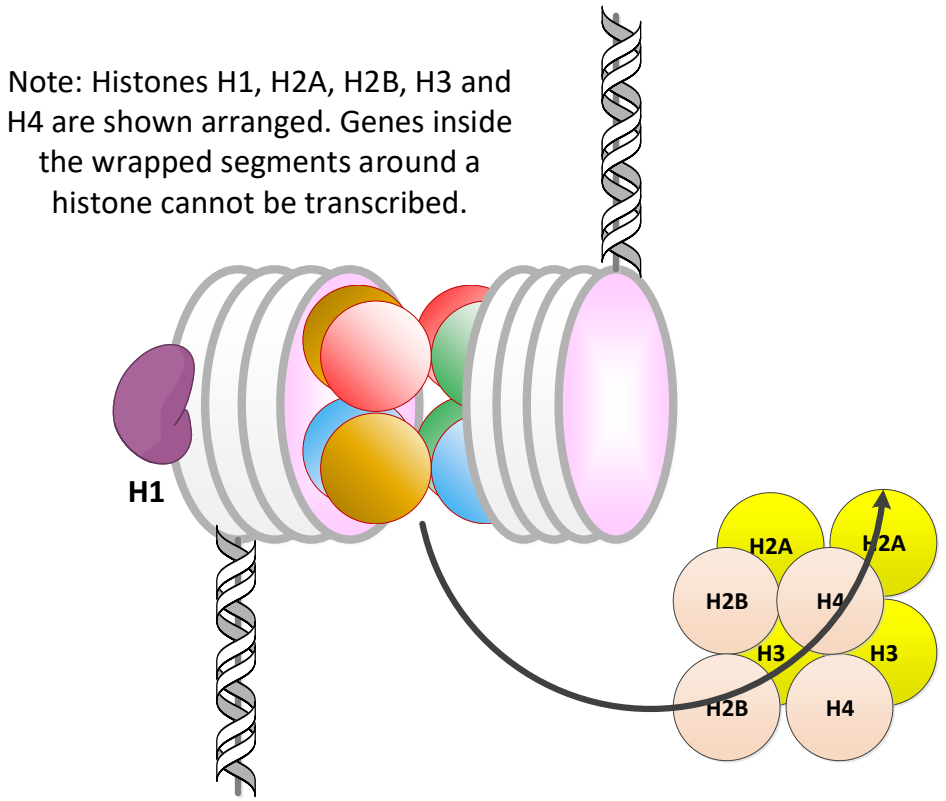
Clearly the ERG fusion plays a significant role in PCa. The AR effects are critical and the overall ETS pathway architecture is also a controlling element. However there is no clear and well defined path and the mechanism for the fusion seems also to be now understood at this time.

4 HISTONES, METHYLATION AND DNA

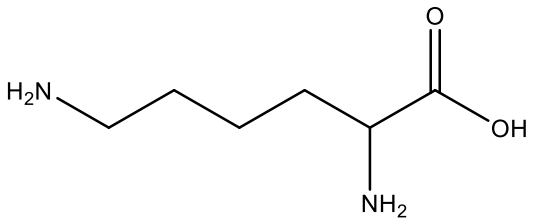
Histone methylation, acetylation and other similar processes dramatically impact the operation of genes. The DNA is wrapped around the histone and is thus unreadable. The histone must facilitate the unwrapping and thus open the DNA for transcription. The transcription factor must then bind in the appropriate spots and then permit the transcribing into an RNA. We briefly discuss these factors and how they relate to ERG and the fusion gene.

4.1 HISTONES

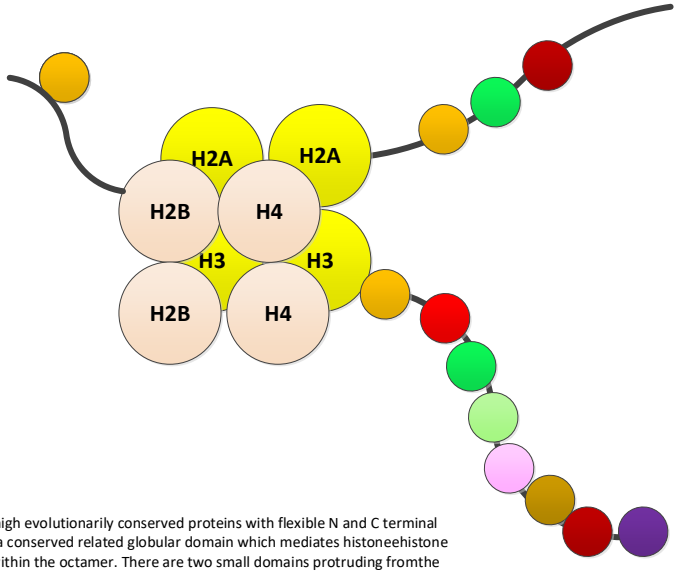
Cellular DNA is not stretched out but tightly wrapped around histones, a collection of eight protein "balls" which allow the DNA to be greatly compressed. In the graphic below we demonstrate this concept. The histones (H2A, H2B, H3, H4) are shown clustered with DNA wrapped around it. Specifically 147 base pairs are wrapped around. Between these histone clusters may be strands of non-clustered DNA. In a cluster the DNA cannot be accessed for reading and for the creation of mRNA. Thus histone sections can be viewed as silencing sections.



Now each of these histone elements have tails of amino acids such a lysine, "K". Lysine is shown below:



Thus the histone tails can be seen as below where lysine and other amino acids are extended beyond the body of the histone.



Histones are high evolutionarily conserved proteins with flexible N and C terminal domains and a conserved related globular domain which mediates histoneehistone interactions within the octamer. There are two small domains protruding fromthe globular domain: an aminoterminal domain constituted by 20e35 residues rich in basic amino acids and a short protease accessible carboxyterminal domain [9e11]. Histone H2A is unique among the histones having an additional 37 amino acids carboxy-terminal domain that protrudes from the nucleosome

Now these tails may be affected by various chemical structure such as methyl like elements. When that happens the normal working may change. In addition these amino acids may already be methylated and this a protein may attack the methylation also changing the characteristics of the specific DNA within it.

4.2 METHYLATION

Methylation is one of the several epigenetic processes that can result in changes in gene expression. Methylation can suppress or activate expression via multiple paths. We briefly examine two of them; DNA and histone effects. It should be noted that methylation also can be accompanied by acetylation and other such factors each of which has their own effects.

DNA methylation is a process whereby the cytosine is changed by the insertion of a methyl group on the 5 carbon of the ring. It is a process which is epigenetic and can dramatically modify gene expression. In fact many of the methylation issue in humans are also common to plants, see the work by Zilberman. There has been a great deal of work demonstrating the impact of methylation on cancer progression; specifically the recent summary by Herman and Baylin, that of Palii and Robertson, that of Robertson and Wolffe, Strathdee and Brown, Calin and Croce, are all worth reviewing.

In this Chapter we examine methylation and its impact on several cancers. We will also examine briefly the causes of methylation as well as the therapeutics in use to modulate cancers that cause or persistence is supported by methylation related products, either directly or indirectly.

In the paper by Das and Singal, the authors define epigenetics in a quite clear manner:

Epigenetics can be described as a stable alteration in gene expression potential that takes place during development and cell proliferation, without any change in gene sequence.

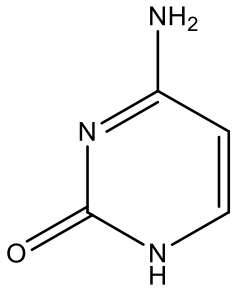
DNA methylation is one of the most commonly occurring epigenetic events taking place in the mammalian genome. This change, though heritable, is reversible, making it a therapeutic target.

Epigenetics has evolved as a rapidly developing area of research.

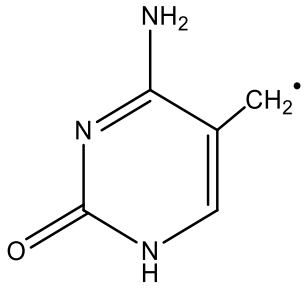
Recent studies have shown that epigenetics plays an important role in cancer biology, viral infections, activity of mobile elements, somatic gene therapy, cloning, transgenic technologies, genomic imprinting, developmental abnormalities, mental health, and X-inactivation

This is one of the clearest definitions of epigenetics and especially the linking of methylation to epigenetics. The classic Watson and Crick model, now some 70 years old, we had the paradigm of DNA, RNA and protein. It was the proteins which did the work. In the 1953 world the proteins stood one by one and the clarity of gene to protein was unquestioned. Yet as we have come to better understand the details, and the details always count, there are many interfering epigenetic factors that all too often get in the way. Methylation is but one of those factors.

Basic cytosine is shown below. It has two NH groups at opposite poles and single oxygen.



Now when the 5 carbon is replaced by a methyl group we obtain the form below. This is methylated cytosine.



Thus this small change in C, by adding the methyl group, can make for a dramatic difference in the expression of genes. For example a well-controlled gene for proliferation, such as PTEN, may have its control over-ridden by the methylation of Introns of CpG islands, namely collections of C, cytosine nucleotides, and G, guanine nucleotides. The introns may be down from the gene, they may even be on a promoter section. The impact could aberrant cell proliferation and growth.

We examine the process; we then look at three types of cancers, a glandular, an epidermal, and a hematopoietic form and then examine some means used to control those cancers through the understanding or methylation and the control of it by therapeutics designed just for that purpose.

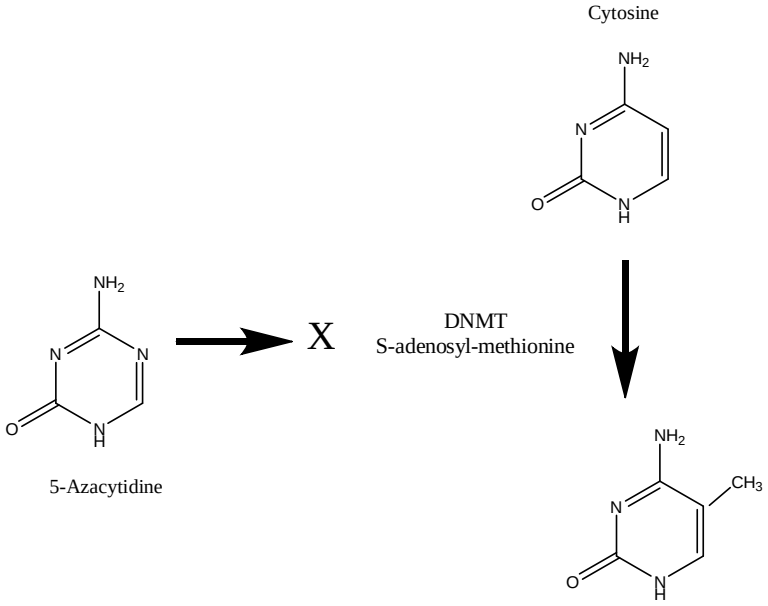
What is important about understanding methylation and especially all epigenetic changes is that it may perhaps be simpler to control them rather than a gene mutation. As Brower states:

The move from a purely genetic to an epigenetic model is crucial for prevention strategies. As numerous gene therapy trials have shown, it is very difficult to treat a genetic disease by re-activating the dormant, mutated gene or by replacing it with a non-mutated one. "Epigenetic changes are reversible, and therefore have an edge over genetics," says Mukesh Verma, an epigeneticist at the National Cancer Institute's division of cancer control and population sciences in Bethesda, Maryland. Furthermore, epigenetic changes in cancer occur before genetic mutations. "If you can prevent methylation of those tumour suppressor genes, you might have a valuable prevention strategy," says Baylin.

Thus if we see cancers when they are driven by methylation, then can we actually anticipate reversing the process by reversing the methylation changes. Thus with prostate cancer can we anticipate a preventative measure as one increasing certain methylation preventative therapeutics, can we do the same with say MDS, and can we attempt to do the same with say a melanoma. This is what we examine herein.

What is methylation? Simply, the attachment of a methyl group to the cytosine molecule creates a methylated C. This is not a complicated process but one which happens frequently and may have significant effects. Cytosine gets methylated and is converted to 5-methyl cytosine. This is accomplished by means of two enzymes as depicted below. This occurs when we have a C and G

adjacent. It occurs to the C in that pair. We depict that transition below. Note also that by using 5-Azacytadine we can block that transition.



From: Herman and Baylin, NEJM,

5-Methyl Cytosine

Now there are the CpG islands. These are C, cytosine, and G, guanine, adjacent nucleotides which are connected via a phosphodiester bone between the two, and multiple collections of these paired nucleotides. The CpG island is then an area dense in these CG pairs connected by the phosphodiester bond, but the "island" may contain nucleotides other than the CG pairs, but generally are high in CG pair concentration, usually more than 50%.

One should note that the statistical probability of such large CG pairings would normally be quite low. One would anticipate equal probability for any nucleotide and any nucleotide pairing. Furthermore such a high concentration is statistically extremely rare but if often existentially quite common.

The CpG islands may be from 300 to over 3,000 base pairs in total length, and are frequently found in gene promoter regions. Thus when the CpG islands are methylated, namely the C is methylated, then the island gets silenced as does the corresponding gene. Namely methylation of CpG islands can result in gene silencing. This then becomes a critical issue if the gene is a control gene such as PTEN, p53, or many of the critical pathway control genes. The CpG islands are also propagated to cell progeny during mitosis, thus a methylated island remains so in the cells progeny.

However understanding methylation of islands, and having a means to demethylate the islands may present a reasonable way to develop therapeutics for cancers resulting from methylated regions. We shall examine that shortly.

As Laird and Jaenisch state:

The normal pattern of 5-methylcytosine distribution DNA methylation in mammals is found as a covalent modification at the fifth carbon position of cytosine residues within CpG dinucleotides. Most of the CpG dinucleotides in the human genome are methylated.

However, 5-methylcytosine makes up less than 1% of all nucleotides, since CpG dinucleotides are under-represented about five-fold in the mammalian genome. The paucity of CpG dinucleotides in the mammalian genome is attributed to a higher mutation rate of methylated versus unmethylated cytosine residues.

CpG dinucleotides and 5-methylcytosine are unevenly distributed in the genome. Most of the genome is heavily methylated with a corresponding deficit in CpG dinucleotides. About 1 to 2% of the genome consists of islands of non-methylated DNA and these sequences show the expected frequency of CpG dinucleotides.

CpG islands are about 1 kb long and are not only CpG-rich, but generally G/C-rich as well and are found at the 5' end of genes. All known housekeeping genes and some tissue-specific genes have associated CpG islands.

We now want to discuss methylation and gene expression. Reference will be made to the work of Herman and Baylin, Jones and Takai, McCabe et al, Allis et al, and Issa and Kantarjian.

We begin with Herman and Baylin and their description of the diagram below:

In most of the mammalian genome, which is depicted here as exons 1, 2, and 3 of a sample gene (boxes 1, 2, and 3), introns of the gene (line between the exons), and regions outside the gene, the CpG dinucleotide has been depleted during evolution, as shown by the small number of such sites (circles).

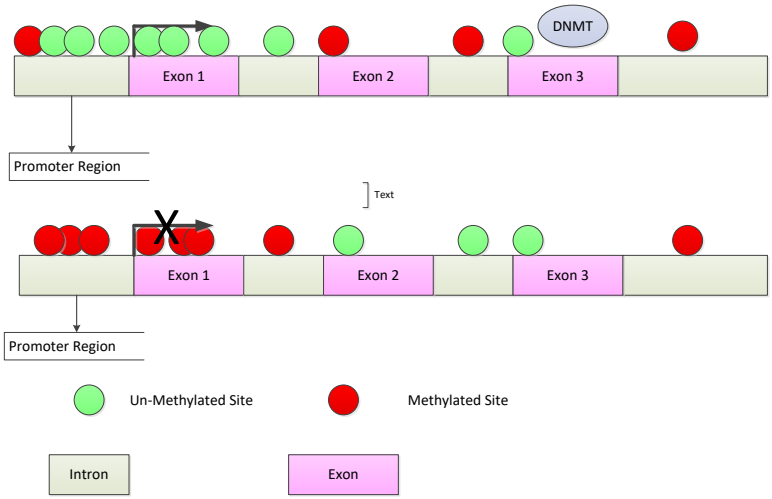
Small regions of DNA, approximately 0.5 to 4.0 kb in size, harbor the expected number of CpG sites and are termed CpG islands. Most of these are associated with promoter regions of approximately half the genes in the genome (numerous circles surrounding and within exon 1 of the sample gene). In normal cells, most CpG sites outside of CpG islands are methylated (black circles), whereas most CpG-island sites in gene promoters are unmethylated (white circles).

This methylated state in the bulk of the genome may help suppress unwanted transcription, whereas the unmethylated state of the CpG islands in gene promoters permits active gene transcription (arrow in upper panel). In cancer cells, the DNA-methylation and chromatin patterns are shifted.

Many CpG sites in the bulk of the genome and in coding regions of genes, which should be methylated, become unmethylated, and a growing list of genes have been identified as having abnormal methylation of promoters containing CpG islands, with associated transcriptional silencing (red X at the transcription start site).

Although there are possible explanations and findings from ongoing investigations, it is not known why the DNA-methylating enzymes fail to methylate where they normally would and which of these enzymes are mediating the abnormal methylation of CpG islands in promoters.

We depict a modified version of their Figure below:



Thus methylation in this case blocks the expression of the targeted gene. Methylation may also progress to more dramatic changes. We discuss here the change of C to T, a serious change in a DNA base pair which can result in dramatic changes in gene expression.

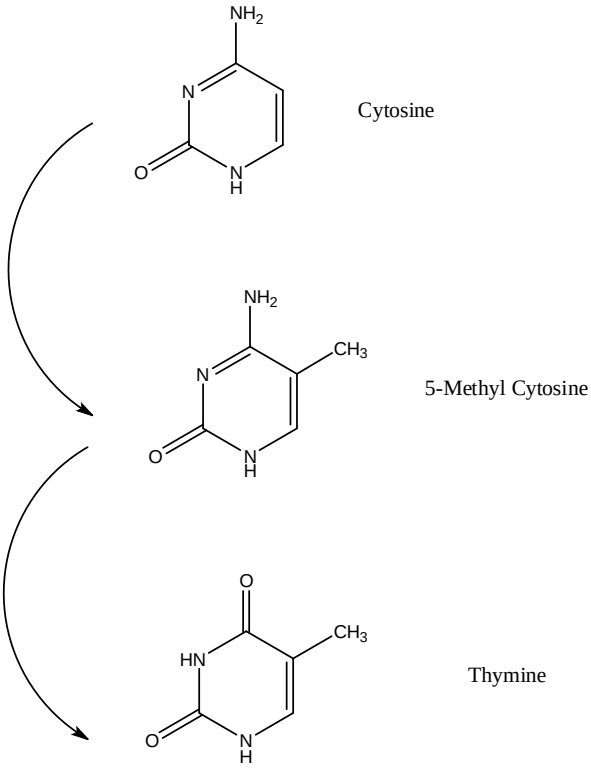
As Herman and Baylin state:

Although only four bases — adenine, guanine, cytosine, and thymine — spell out the primary sequence of DNA, there is a covalent modification of post-replicative DNA (i.e., DNA that has replicated itself in a dividing cell) that produces a "fifth base." Reactions using S -adenosyl-methionine as a methyl donor and catalyzed by enzymes called DNA methyltransferases (DNMTs) add a methyl group to the cytosine ring to form methyl cytosine.

In humans and other mammals, this modification is imposed only on cytosines that precede a guanosine in the DNA sequence (the CpG dinucleotide). The overall frequency of CpGs in the genome is substantially less than what would be mathematically predicted , probably because DNA methylation has progressively depleted the genome of CpG dinucleotides over the course of time.

The mechanism of the depletion is related to the propensity of methylated cytosine to deaminate, thereby forming thymidine. If this mutation is not repaired, a cytosine-to-thymidine change remains.

The depletion of CpG dinucleotides in the genome corresponds directly to sites of such nucleotide transitions, and this change is the most common type of genetic polymorphism (variation) in human populations.



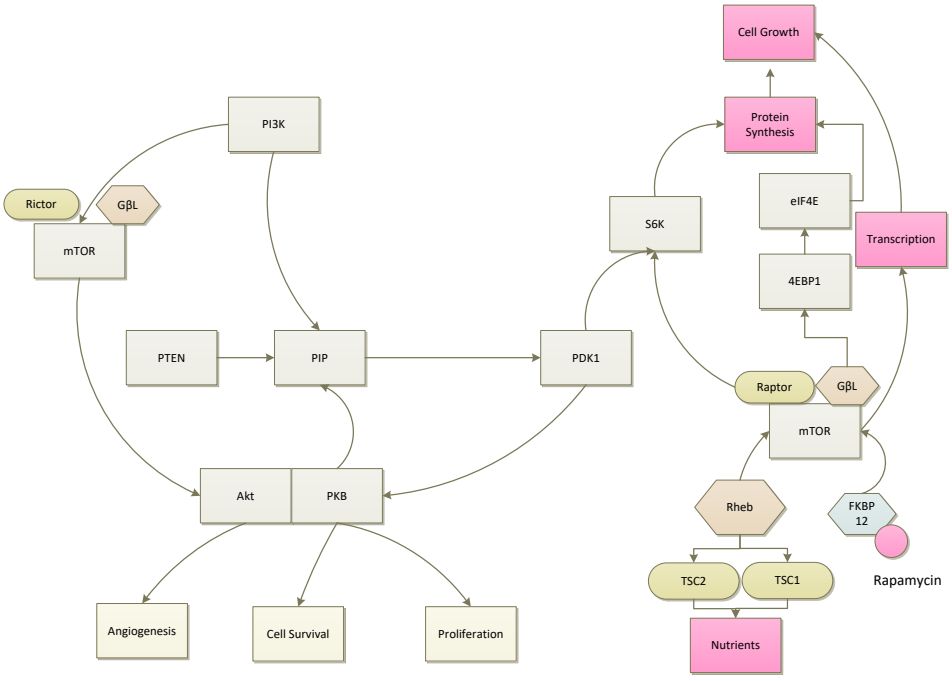
From Robertson (2001) we have some of the genes influenced by methylation or as he states:

CpG-island-associated genes involved in cell growth control or metastasis that can become hypermethylated and silenced in tumors.

We depict the Table below from Robertson on some of the genes impacted by this type of methylation. Most of these are significant regulatory genes.

Gene	*Function*
pRb	Regulator of G1/S phase transition
$p16^{INK4a}$	Cyclin-dependent kinase inhibitor
$p15^{INK4b}$	Cyclin-dependent kinase inhibitor
ARF	Regulator of p53 levels
hMLH1	DNA mismatch repair
APC	Binds β-catenin, Regulation of actin cytoskeleton?
VHL	Stimulates angiogenesis
BRCA1	DNA repair
LKB1	Serine/threonine protein kinase
E-cadherin	Cell - cell adhesion
ER	Transcriptional activation of estrogen-responsive genes
GSTPI	Protects DNA from oxygen radical damage
0^6-MGMT	Repair/removal of bulky adducts from guanine
TIMP3	Matrix metalloproteinase inhibitor
DAPK1	Kinase required for induction of apoptosis by y interferon
p73	Apoptosis structurally similar to p53

For example we show below some typical pathways and the above genes are seen targeted by methylation.



Methylation may then interfere with many of the genes in the above pathways.

The major question which is often asked is what causes methylation. In Allis et al on p 460 the authors discuss some of the putative cause of methylation and methylation related cancers. Although not confirmative it is consistent with clinical correlations as well.

As Issa and Kartarjian state:

Much remains to be learned about the causes of DNA methylation abnormalities in cancer; for the most part, methylation seems to be gene specific. In some cases, a rare methylation event appears in cancer because of selection , while in others methylation anomalies are downstream of an oncogenic event …

As McCabe et al state:

DNA methylation patterns in human cancer cells are considerably distorted. Typically, cancer cells exhibit hypomethylation of intergenic regions that normally comprise the majority of a cell's methyl-cytosine content . Consequently, transposable elements may become active and contribute to the genomic instability observed in cancer cells.

Simultaneously, cancer cells exhibit hypermethylation within the promoter regions of many CpG island-associated tumor suppressor genes, such as the retinoblastoma gene (RB1), glutatione S-transferase pi (GSTP1), and E-cadherin (CDH1). As a result, these regulatory genes are transcriptionally silenced resulting in a loss-of-function. Thus, through the effects of both hypo- and hyper-methylation, DNA methylation significantly affects the genomic landscape of cancer cells, potentially to an even greater extent than coding region mutations, which are relatively rare

McCabe et al continue:

Although the precise molecular mechanisms underlying the establishment of aberrant DNA hypermethylation remain elusive, recent studies have identified some contributing etiologic factors.

For example, chronic exposure of human bronchial epithelial cells to ***tobacco-derived carcinogens drives hypermethylation*** *of several tumor suppressor genes including CDH1 and RASSF2A.*

Stable knockdown of DNMT1 prior to carcinogen exposure prevented methylation of several of these genes indicating a necessary role for this enzyme in the molecular mechanism underlying hypermethylation.

The reactive oxygen species (ROS) associated with chronic inflammation is another source of DNA damage with the potential to affect DNA methylation as halogenated pyrimidines, one form of ROS-induced damage, mimic 5-methylcytosine and stimulate DNMT1-mediated CpG methylation in vitro and in vivo.

Indeed, study of the glutatione peroxidase 1 and 2 double knockout model of inflammatory bowel disease found that 60% of genes that are hypermethylated in colon cancers also exhibit aberrant

methylation in the inflamed noncancerous precursor tissues. Although the mechanisms by which DNA damage mediates DNA methylation are not fully understood, O'Hagan and colleagues have examined the process with an engineered cell culture model in which a unique restriction site was incorporated into the CpG island of the E-cadherin promoter.

Thus the actual molecular mechanics leading to methylation are not fully understood but like most cancers inflammation appears to be a driving factor. What the cause of that inflammation may be is not yet clear.

As is stated in the paper by Miranda and Jones:

DNA methylation is a covalent modification in which the 5_0position of cytosine is methylated in a reaction catalyzed by DNA methyltransferases (DNMTs) with S-adenosyl-methionine as the methyl donor.

In mammals, this modification occurs at CpG dinucleotides and can be catalyzed by three different enzymes, DNMT1, DMNT3a, and DNMT3b.DNAmethylation plays a role in the long-term silencing of transcription and in heterochromatin formation.

As an epigenetic modification, DNA methylation permits these silenced states to be inherited throughout cellular divisions.

We continue with the discussion in Mirand and Jones as follows:

Silencing of genetic elements can be successfully initiated and retained by histone modifications and chromatin structure. However, these modifications are easily reversible making them make poor gatekeepers for long-term silencing. Therefore, mammalian cells must possess an additional mechanism for prolong silencing of these sequences. An important component of this process is DNA methylation. DNA methylation is a stable modification that is inherited throughout cellular divisions.

When found within promoters, DNA methylation prevents the reactivation of silent genes, even when the repressive histone marks are reversed. This allows the daughter cells to retain the same expression pattern as the precursor cells and is important for many cellular processes including the silencing of repetitive elements, X-inactivation, imprinting, and development.

We now present a key Figure from Miranda and Jones regarding the methylated reading of DNA. They state regarding the Figure below:

Chromatin structure of CpG islands and CpG poor regions in healthy cells and during cancer. In healthy cells, CpG islands are generally hypomethylated. This allows for an open chromatin structure. However, the CpG poor regions found in repetitive elements within the intergenic and intronic regions of the genome are methylated and thereby maintain a closed chromatin structure. In cancer and on the inactive X chromosome many CpG islands become methylated, forcing these regions into a closed chromatin structure.

When CpG islands located within promoters are methylated, the corresponding genes are persistently silenced. In contrast, the CpG poor regions become hypomethylated allowing for an open chromatin structure.

As Robertson states:

It is now clear that the genome contains information in two forms, genetic and epigenetic. The genetic information provides the blueprint for the manufacture of all the proteins necessary to create a living thing while the epigenetic information provides instructions on how, where, and when the genetic information should be used.

Ensuring that genes are turned on at the proper time is as important as ensuring that they are turned off when not needed.

The major form of epigenetic information in mammalian cells is DNA methylation, or the covalent addition of a methyl group to the 5-position of cytosine predominantly within the CpG dinucleotide. DNA methylation has profound effects on the mammalian genome.

Some of these effects include transcriptional repression, chromatin structure modulation, X chromosome inactivation, genomic imprinting, and the suppression of the detrimental effects of repetitive and parasitic DNA sequences on genome integrity.

Robertson then proceeds to detail the genes impacted by hypermethylation. We summarize them below:

Gene	*Function*
pRb	Regulator of G1/S phase transition
p16 INK4a	Cyclin-dependent kinase inhibitor
p15 INK4b	Cyclin-dependent kinase inhibitor
ARF	Regulator of p53 levels
hMLH1	DNA mismatch repair
APC	Binds b-catenin, Regulation of actin cyto-skeleton?
VHL	Stimulates angiogenesis
BRCA1	DNA repair
LKB1	Serine/threonine protein kinase
E-cadherin	Cell ± cell adhesion
ER	Transcriptional activation of estrogen-responsive genes
GSTP1	Protects DNA from oxygen radical damage
O6-MGMT	Repair/removal of bulky adducts from guanine
TIMP3	Matrix metallo proteinase inhibitor
DAPK1	Kinase required for induction of apoptosis by g interferon
p73	Apoptosis?, structurally similar to p53

Regarding PIN, the one which is most concern is the GSTP1 gene and its suppression allowing for DNA damage from inflammation and oxygenation damage.

In the context of cancer generation and progression, the epigenetic effect of hyper and hypo methylation is best described by Esteller:

The low level of DNA methylation in tumors as compared with the level of DNA methylation in their normal-tissue counterparts was one of the first epigenetic alterations to be found in human cancer.

The loss of methylation is mainly due to hypomethylation of repetitive DNA sequences and demethylation of coding regions and introns −regions of DNA that allow alternative versions of the messenger RNA (mRNA) that are transcribed from a gene. A recent large-scale study of DNA methylation with the use of genomic microarrays has detected extensive hypo-methylated genomic regions in gene-poor areas.

During the development of a neoplasm, the degree of hypomethylation of genomic DNA increases as the lesion progresses from a benign proliferation of cells to an invasive cancer.

Three mechanisms have been proposed to ex-plain the contribution of DNA hypomethylation to the development of a cancer cell:

(i) generation of chromosomal instability,

(ii) reactivation of transposable elements, and

(iii) loss of imprinting.

Under methylation of DNA can favor mitotic recombination, leading to deletions and translocations, and it can also promote chromosomal rearrangements. This mechanism was seen in experiments in which the depletion of DNA methylation by the disruption of DNMTs caused aneuploidy. Hypomethylation of DNA in malignant cells can reactivate intra-genomic endo-parasitic DNA.

4.2.1 Hypomethylation

As Laird and Jaenisch state:

Hypomethylation: Reduced levels of global DNA methylation have been reported for a variety of malignancies in the past decade. Gama Sosa and coworkers found that in a wide variety of tumors, hypomethylation not only correlated with transformation, but also with tumor progression . In their analysis, only 7% of 43 normal tissues had a 5-methylcytosine content below 0.8 mol%, whereas 10% of 21 benign tumors, 27% of 62 primary malignancies and 60% of 20 secondary malignancies had a 5-methylcytosine content below 0.8 mol%. On the other hand, Feinberg and coworkers did not find a further reduction in DNA methylation levels in the progression from benign to malignant colonic neoplasia, suggesting an early role for DNA hypomethylation in colorectal cancer

4.2.2 Hypermethylation

As again with Laird and Jaenisch we have:

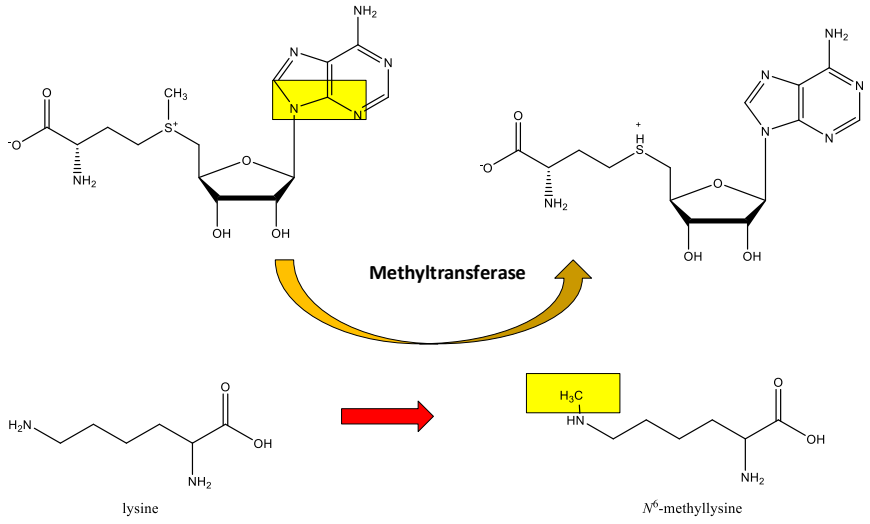
Hypermethylation: There have also been many reports of regional increases in DNA methylation levels. Baylin and coworkers have found regional hotspots for hypermethylation on chromosomes 3p, 11p and 17p in a variety of human tumors. These include CpG island areas that are normally never methylated in vivo, but are found to be methylated in tumor tissues. This is reminiscent of the changes that occur at CpG islands at non-essential genes in tissue culture. Baylin's group has dissected the sequential order of hypermethylation events in an in vitro model for lung tumor progression.

There is evidence for inactivation of tumor-suppressor gene function through hypermethylation of the Rb gene in sporadic retinoblastoma. Transient transfection experiments showed that specific hypermethylation in the promoter region of Rb could reduce expression to 8% of an unmethylated control. It is possible, therefore, that hypermethylation of tumor-suppressor genes leading to gene inactivation results in a selective growth advantage of the transformed cells.

4.3 HISTONE METHYLATION

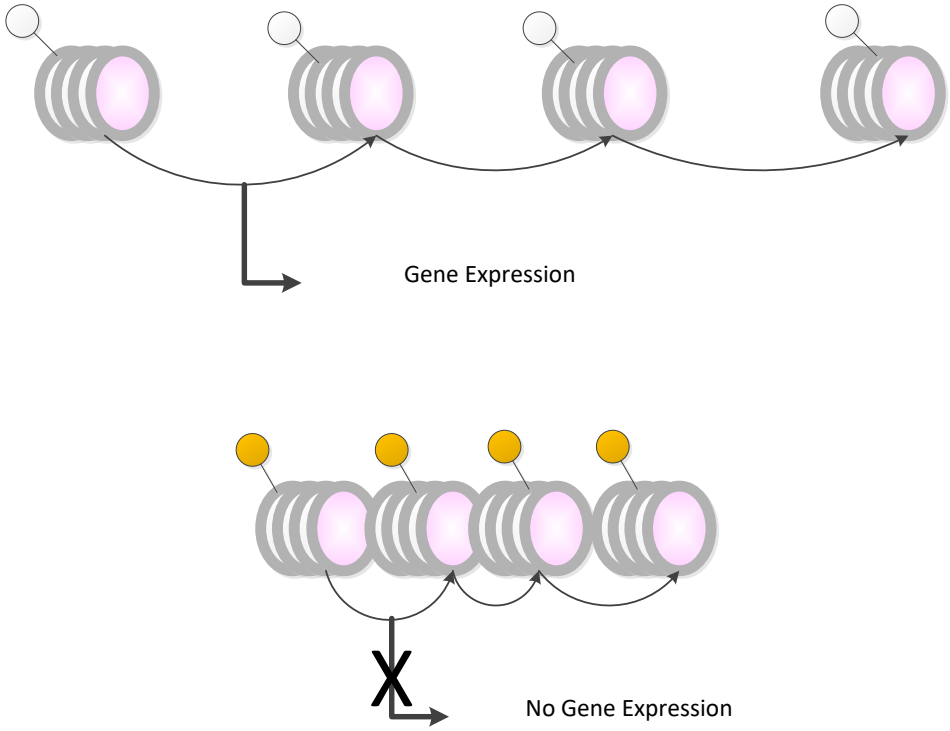
Histones can also be methylated. Here the effects can be materially more complex. The methylation can occur on the tails sticking out and each histone element has a tail and thus there interaction can be amongst the same histone tail or even more so between adjacent histone tails. This interaction results in the opening and closing of DNA, making for the expression or suppression of many genes.

A typical methylation is enabled by an enzyme called a methyltransferase. We show this below.



Namely the lysine on a tail can be methylated on the distal end by transferring the methyl group using the enzyme methyltransferase. Lysine is not methylated and as such as acquired a substantially different set of attachment properties. A similar process can occur with acetylation. These processes are also reversible.

Now when we examine a histone complex it may appear as follows:



The top part shows no methylation and as such we have the DNA open between histone clusters. Now if we were to methylate them we may see the case shown below it, as above, where now we cannot insert a transcription factor and other necessary elements to effect transcription. Thus

As Helin and Dhanak note:

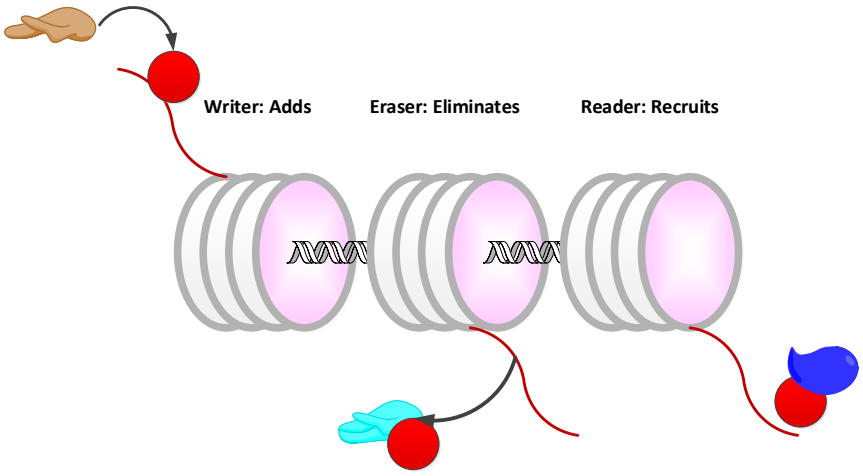
DNA is wrapped around histones (H2A, H2B, H3 and H4) to form nucleosomes. Nucleosomes are further compacted to form condensed chromatin. The compaction of DNA is in part regulated through post-translational modifications (PTMs) of the histone tails, which protrude from nucleosomes. Epigenetic regulators can in popular terms be divided into erasers, writers or readers of PTMs.

The erasers, such as histone deacetylases and histone demethylases, remove the PTMs and prepare the histones for other modifications.

The writers comprise enzymes such as histone acetylases, kinases, DNA and histone methyltransferases and ubiquitin ligases. The writers catalyse the PTMs on the DNA or the proteins, and may impose epigenetic heritability such as DNA methylation through copying and maintaining the modification.

Other modifications, such as histone acetylation, respond rapidly to environmental stimuli and are therefore more dynamic.

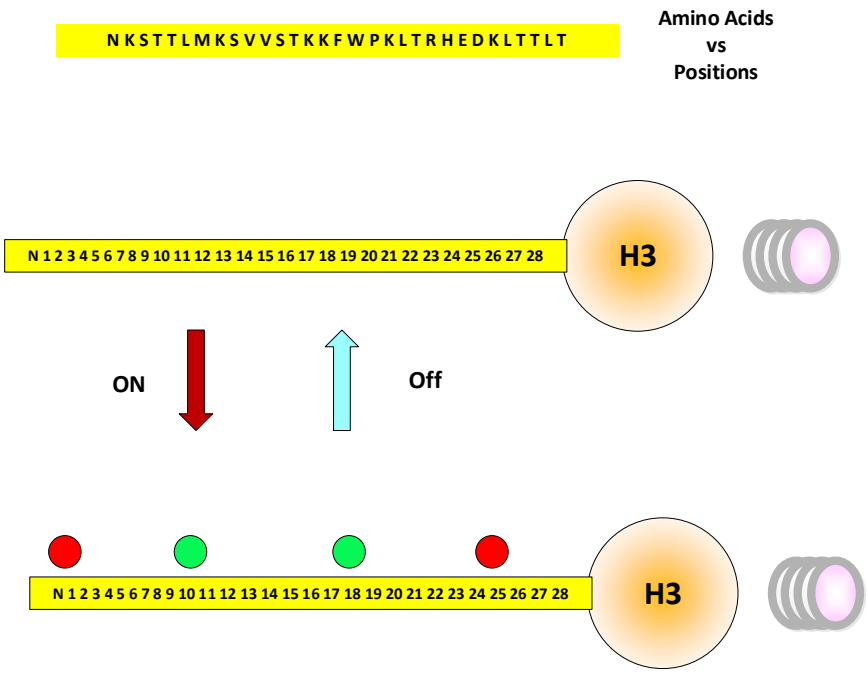
Readers of the post-translational modification include proteins with specific domains, such as bromo-, chromo-, tudor-, MBT-, PWWP-, WD40- and PHD-domains, which bind to the specific modification. The readers, which are often found in large protein complexes, interpret the modification and impose changes in chromatin structure.



The above depicts graphically these three processes. The work by Gut and Verdin further discuss this process as a temporal one, inherent in ageing of the genetic environment.

4.4 THE HISTONE CODE

The Histone Code was described by Strahl and Allis in 2000 and it can be simply explained as follows.



In the above we have a tail and tail locations and respective amino acids for each location. Now on the top there are no methylations or acetylations. We have then done so on the one below. We can assert that in the top condition we have the base state and then the one below some active state. Thus we go from off to on whatever that may mean. Thus as Strahl and Allis note in their presentation we have:

N	*1*	*2*	*3*	*...*	*27*	*28*	*Modification State*	*Associated Protein*	*Function*
	M						Methylated	SIRT	Silencing
		M					Methylated	SMC	Transcription
				M	M		Methylated	RCAF	Mitosis
	A						Acetylated	Bromodomain	Transcription
		A		P	M		Complex	TWIST	Silencing

Namely the histone code postulates what reaction will ensue when we have some form of epigenetic change on a specific tail of a specific histone and it indicates what protein is necessitated to effect this epigenetic change.

Now the histone code relates to the state of the tail as described by methylations or other related attachments and the resulting actions related thereto.

The above demonstrates the tail composed of a collection of amino acids and the extension of that from each of the histone elements. These tails allow for reactions which in turn result in changes of gene expression. As we shall see, the protein we are focusing on, NSD2, is a histone modifying protein and it targets a specific amino acid on the histone. In this case it targets H3K36me3. This nomenclature states:

1. Histone H3
2. K for lysine

3. Location 36 on the tail
4. methylated
5. tri methylated

Thus the notation can be specific as to the tuple:

{*histone:amino acid:location:modification:degree*} =- H3K36me3.

As Jenuwein and Allis had noted in 2000:

Chromatin, the physiological template of all eukaryotic genetic information, is subject to a diverse array of posttranslational modifications that largely impinge on histone amino termini, thereby regulating access to the underlying DNA. Distinct histone amino-terminal modifications can generate synergistic or antagonistic interaction affinities for chromatin-associated proteins, which in turn dictate dynamic transitions between transcriptionally active or transcriptionally silent chromatin states. The combinatorial nature of histone amino-terminal modifications thus reveals a "histone code" that considerably extends the information potential of the genetic code.

From Tollefsbol we have:

Equally important in the fine tuning control of chromatin organization is the interplay between the histone modifications, DNA methylation and ATP-dependent chromatin remodeling. The large number of histone modifications and the possible interplay between them led to the proposition of the so-called "histone code hypothesis" in which "multiple histone modifications, acting in a combinatorial or sequential fashion on one or multiple histone tails, specify unique downstream functions".

This hypothesis led the scientific community to adopt some metaphors to describe it such that the code is written by some enzymes ("writers"), removed by others ("erasers"), and is readily recognized by proteins ("readers") recruited to modifications through the binding of specific domains.

Such a simplified version of the code is depicted below. Here we have depicted it differently. The top row is the histone element, the column the change which is made, and the cell entity is what happens when that change in epigenetic structure is made. This of course is a highly simplified result.

	H3K4	H3K9	H3K14	H3K27	H3K79	H4K20	H2BK5
Mono-meth	Active	Active		Active	Active	Active	Active
Di-meth		Repress		Repress	Active		
Tri-meth	Active	Repress		Repress	Active		Repress
Acetyl		Active	Active		Repress		

More complicated versions are available, In 2000 Strahl and Allis noted:

The `histone code' hypothesis. Histone modifications occur at selected residues and some of the patterns shown have been closely linked to a biological event (for example, acetylation and transcription). Emerging evidence suggests that distinct H3 and H4 tail modifications act sequentially or in combination to regulate unique biological outcomes. How this hierarchy of multiple modifications extends (depicted as `higher-order combinations') or how distinct combinatorial sets are established or maintained in localized regions of the chromatin fiber is not known.

Relevant proteins or protein domains that are known to interact or associate with distinct modifications are indicated. The CENP-A tail domain might also be subjected to mitosis-related marks such as phosphorylation; the yellow bracket depicts a motif in which serines and threonines alternate with proline residues

From Tollefsbol (see Fig 4.1 Chapter 4) we have another slightly more complicated version:

Chromatin Modification	***Residues modified***	***Function regulated***
Acetylation	Lysine	Transcription, DNA repair, replication and condensation
Methylation (Lysine)	Lysine me1, me2, me3	Transcription, DNA repair
Methylation (Arginine)	Arginine-me1, Arginine-me2a Arginine-me2s	Transcription
Phosphorylation	Serine, Threonine, Tyrosine	Transcription, DNA repair and condensation
Ubiqutination	Lysine	Transcription, DNA repair
Sumoylation	Lysine	Transcription
ADP ribosylation	Glutamic	Transcription
Deimination	Arginine	Transcription
Proline isomerization	P-cis, P-trans	Transcription

In summary we can articulate this as follows:

1. A base state is present and in the base state the genes follow the base state expression.

2. A methyltransferase or equivalent is introduced. This means that it is activated by some means. We leave that to the side for the moment.

3. The methyltransferase targets a specific histone tail element. It then methylates that element.

4. The methylated tail then reconfigures the histone arrangement, opening or closing sections of DNA.

5. DNA expression is altered as a result of the change in the histone configurations. Proteins are produced which are then sent from the nucleus or kept there.

6. The new proteins commence the actions for which they function. Cells then proliferate, go through epithelial-mesenchymal transitions and the like.

Conceptually this is a simple process but in actuality there are a multiplicity of questions as to what and why.

4.5 IMPLICATIONS

Now Li et al have noted that histone modification via ERG can result in differentially expressed genes ("DEG"). DEGs are genes whose expression are significantly modified during certain situations. In Li et al analysis there were a multiple DEGs observed in the PCa studies, ERG being a significant one. As they note:

TMPRSS2-ERG translocation represents a distinct subset on the cis-regulatory landscape in primary prostate tumors. ***ERG overexpression was known to induce the global changes in chromatin conformation.*** *Here, we have further proved that ERG overexpression globally induces chromatin interaction changes. Moreover, these chromatin interaction changes are associated with the coordinated DEG expressions. Through a distant binding,*

ERG can regulate Trp expression by chromatin interactions. Importantly, deletion of this binding site remarkably reverses the lineage plasticity towards basal differentiation. Compelling data in supporting this hypothesis has also been obtained from the re-analysis on the publicly available human datasets with ERG ChIP-seq, which can validate the conserved existence of ERG binding site in human prostate cells. Therefore, we have successfully obtained a novel finding of the conserved ERG binding site that contributes to prostate lineage plasticity. In addition, we have also provided a novel research paradigm for the investigation on how TFs regulate their responsive genes through chromatin interactions instead of direct binding at the gene body regions. …

ERG overexpression driven by TMPRSS2-ERG fusion is one of the most common genetic alteration events in prostate cancer, which can alter chromatin interactions*. Since chromatin architecture is closely associated with epigenetic modifications and mRNA transcription,* ***ERG-induced alterations in chromatin interactions may cause dysregulation of genes including Trp63. ERG overexpression reduces chromatin interactions and H3K27ac levels across the region from a distal ERG binding site to Trp63 gene body, which further causes decreased mRNA levels of Trp63 to facilitate the function of ERG in promoting luminal lineage differentiation***

This is a significant observation. It links gene expression with epigenetic modification via chromatin loosing.

Recent work by Kin et al have also noted histone control in PCa. They note:

In the present study, we uncovered a new mechanism by which ERG may exert its oncogenic function. This mechanism involves a physical interaction of ERG with the histone demethylase KDM4A that could lead to pleiotropic changes in the transcriptome, including an upregulation of YAP1 gene transcription. Since ERG overexpression is found in approximately half of all prostate tumor patients, our findings particularly pertain to prostatic malignancies.

YAP1 is a transcriptional cofactor that can be recruited to chromatin by several DNA-binding proteins[11]. Frequently, YAP1 expression is enhanced in various human tumors and may correlate with poor prognosis, and its oncogenic potential was confirmed both in vitro as well as in transgenic mouse models. However, recent studies suggest that YAP1 may also exert growth suppressive actions in the colon and hematological cancers, suggesting that YAP1 context-dependently acts as an oncogene or tumor suppressor.

However, the fact that YAP1 is overexpressed in human prostate tumors indicates that it functions as an oncogene in this organ, which is consistent with prostate-specific overexpression of YAP1 leading to the development of prostatic neoplasias in mice. All this stresses that YAP1 may serve as a target for therapy particularly in ERG-overexpressing prostate tumors. Notably, small molecules as well as a peptide that suppress YAP1 function have been identified, which could be harnessed for future avenues of therapeutic interference.

A caveat is that our report does not establish whether YAP1 is the only crucial downstream effector of ERG. Given that ERG downregulation seems to be more detrimental to VCaP cell proliferation than YAP1 downregulation, it is likely that YAP1 upregulation is not the sole reason why ERG overexpression induces prostate tumors. Yet, even partially blunting ERG's oncogenic potential through YAP1 inhibition would still have therapeutic value.

KDM4A is the protagonist of the KDM4 family of histone demethylases that are encoded by six different genes in the human genome. It is particularly competent in demethylating trimethylated lysine 9 on histone H3 and lysine 26 on histone H1.4 that are regarded as repressive chromatin marks (46,47,56,57). Accordingly, KDM4A may function as a transcriptional coactivator at least in part by removing these repressive marks. However, we observed that catalytically inactive KDM4A was still capable, albeit at a much reduced rate compared to wild-type KDM4A, to cooperate with ERG in stimulating the YAP1 promoter.

This suggests that KDM4A coactivates ERG both in a manner dependent on and independent of its catalytic activity. Likewise, Drosophila KDM4A has been shown to often affect gene transcription independent of its catalytic activity and also mammalian KDM4A can impact DNA

[11] https://www.ncbi.nlm.nih.gov/gene/10413 *This gene encodes a downstream nuclear effector of the Hippo signaling pathway which is involved in development, growth, repair, and homeostasis. This gene is known to play a role in the development and progression of multiple cancers as a transcriptional regulator of this signaling pathway and may function as a potential target for cancer treatment.*

repair without involving its catalytic activity, corroborating that KDM4A may act both as an enzyme and in non-enzymatic ways.

However, in case of stimulating ERG, our data suggest that KDM4A is mostly acting through its enzymatic activity. If so, inhibition of its catalytic center may prove beneficial in the treatment of prostate cancer patients that are afflicted by an ERG chromosomal translocation. Several small molecules have been uncovered that can inhibit KDM4A enzymatic activity (60–66). However, the specificity of these inhibitors, their selectivity for suppressing tumor vs. normal cells, their toxicity, pharmacokinetics and pharmacodynamics need to be further explored before any of these inhibitors can enter clinical trials.

Similar to ERG, KDM4A seems to be overexpressed in prostate tumors (67), which would be alike to breast and lung tumors that display overexpression of KDM4A (68–71). This may suggest that KDM4A is oncogenic in its own right in the prostate, breast or lung. Furthermore, it is unlikely that KDM4A exclusively promotes prostate tumorigenesis as a coactivator of ERG. For instance, KDM4A can also stimulate the androgen receptor or repress the p53 tumor suppressor thereby leading to abnormal cell growth (72,73). Moreover, KDM4A is capable of inducing copy number gains in cells, which may represent another mechanism by which it contributes to the development of cancer (74).

In conclusion, the present study has provided more mechanistic insight into how ERG overexpression due to chromosomal translocations can induce prostate cancer formation. Despite its obvious validity as a drug target in prostate cancer, no effective ERG inhibitors have surfaced in the clinic, which may be due to the difficulty of targeting a DNA-binding transcription factor. The present study suggests two alternative targets to blunt the ERG oncogenic activity, KDM4A and YAP1, both of which can in principal be inhibited by small molecules and may therefore merit more research.

5 RECENT OBSERVATIONS

We return to the recent paper by Li and examine their observations and conclusions. The authors start by asserting:

Identification on the master transcription factors has provided significant insights to understand both 4 plasticity of prostate cancer lineages and mechanism of therapy resistances. For example, N-Myc was 5 identified as an oncogenic driver to promote neuroendocrine prostate cancer differentiation in the context 6 of PI3K pathway activation in both GEM mouse models () and transformation cellular models of human 7 prostate epithelial cells.

In addition, SOX2 was recognized as a key transcription factor to facilitate 8 the lineage transitions from prostate luminal cell lineage to neuroendocrine and basal cell lineage in TP- 9 deficient and RB1-deficient GEM mouse models as well as cellular models of human prostate cancer cell lines. Together, these findings proposed that SOX2 played a vital and context-dependent role for regulation on prostate cancer lineages. SOX, as another member of SOX gene family, also promoted neuroendocrine differentiation and the treatment resistance to prostate cancers in the context of PTEN and TP inactivation.

Given that the advanced prostate cancer lineage is predominantly regulated by these known transcription factors, it is reasonable to question that how primary prostate cancers gain their luminal differentiation features.

The term "master transcription factor" is of interest. They assert:

Tumor initiation, progression, and therapy resistance involve epigenetic reprogramming that leads to aberrant cell lineage specification and transition. It is critical to understand the underlying mechanisms of cancer cell lineage differentiation and transition, which will provide novel insights into anticancer research. Master transcription factors have been widely recognized with the function in cell lineage trans-differentiation and cell fate reprogramming. The identification of master transcription factors in regulation cancer cell lineage specification and transition would provide tremendous insights into the mechanism of lineage plasticity in cancer progression and therapy resistance

As Whyte et al note:

Master transcription factors Oct4, Sox2 and Nanog bind enhancer elements and recruit Mediator to activate much of the gene expression program of pluripotent embryonic stem cells (ESCs). We report here that the ESC master transcription factors form unusual enhancer domains at most genes that control the pluripotent state. These domains, which we call super-enhancers, consist of clusters of enhancers that are densely occupied by the master regulators and Mediator. Super enhancers differ from typical enhancers in size, transcription factor density and content, ability to activate transcription, and sensitivity to perturbation.

Reduced levels of Oct4 or Mediator cause preferential loss of expression of super-enhancer-associated genes relative to other genes, suggesting how changes in gene expression programs might be accomplished during development. In other more differentiated cells, super-enhancers containing cell type-specific master transcription factors are also found at genes that define cell identity. Super-enhancers thus play key roles in the control of mammalian cell identity. Transcription factors typically regulate gene expression by binding cis-acting regulatory elements known as enhancers and recruiting coactivators and RNA Polymerase II (RNA PolII) to target genes. Enhancers are segments of DNA that are generally a few hundred base pairs in length and are typically occupied by multiple transcription factors

Now Li et al conclude:

1. ERG regulates normal prostate epithelial cell lineage

2. ERG regulates prostate cancer cell lineage

3. ERG but not AR is sufficient to maintain luminal lineage in Pten loss prostate cancer

4. ERG induces the global changes in chromatin interactions

5. Deletion of a specific ERG binding site disrupts the function of ERG in prostate lineage regulation

The authors overall conclusions are further specified as follows:

Definitive evidence collected during past years supports the close associations between activity of transcription factors (TFs) and cell lineage determination in various biological processes, including development, immune response and cancer progression. Particularly, primary prostate cancer is characterized with both luminal cells expansion and loss of basal cells. Therapeutic treatments on prostate cancers can select for lineage alterations with the transitions from luminal cell lineage toward neuroendocrine and basal differentiation. Numerous studies have focused on lineage transitions in CRPC. However, the lineage determining mechanism of primary prostate luminal cancers are still largely unknown.

Here, we have successfully identified ERG as a master regulator in regulating prostate cancer cell luminal lineage through chromatin interaction changes. TMPRSS2-ERG fusion is a common genetic alteration event which drives ERG expression occurring in the early-stage of prostate cancer. We identified ERG as a master regulator in prostate cancer lineage regulation through the integrating analysis of three high-quality human prostate cancer cohorts.

It is widely accepted that both prostate basal and luminal cells have bi-potential plasticity, which was found in 3 dimensional organoids and UGSM tissue recombination assay. In this study, we found that ERG expression strongly facilitates the differentiations towards luminal phenotype in both luminal organoids and basal organoids, consistent with previous findings that ERG expressions induced a significant decrease in the proportion of prostate basal cells.

Moreover, our current study indicates that luminal cells tend to be more liable for lineage regulation conducted by ERG, when compared with basal cells. Together with their clinical relevance, our findings suggest the important role of ERG in initiation of primary prostate cancer with luminal cell features*.*

Previous studies have provided some insights into the functional role of androgen receptor (AR) in cell lineage regulation in both normal prostate development and prostate cancer. In vivo tissue recombination modeling suggests that stromal AR, but not epithelial AR, is essential for prostate developmental growth and morphogenesis.
…

Therefore, further researches to define other master regulators with the function in ERG-negative prostate cancer or normal prostate lineage regulation are warranted, which may provide rationale for a novel therapeutic strategy and prostate development. Further investigations to dissect cancer-stage-specific roles of luminal-cell AR in both primary prostate cancer and advanced prostate cancer will be really necessary.

…TMPRSS2-ERG translocation represents a distinct subset on the cis-regulatory landscape in primary prostate tumors. ERG overexpression was known to induce the global changes in chromatin conformation. Here, we have further proved that ERG overexpression globally induces chromatin interaction changes.

Moreover, these chromatin interaction changes are associated with the coordinated DEG expressions. *Through a distant binding, ERG can regulate Trp expression by chromatin interactions. Importantly, deletion of this binding site remarkably reverses the lineage plasticity towards basal differentiation. Compelling data in supporting this hypothesis has also been obtained from the re-analysis on the publicly available human datasets with ERG ChIP-seq, which can validate the conserved existence of ERG binding site in human prostate cells (). Therefore, we have successfully obtained a novel finding of the conserved ERG binding site that contributes to prostate lineage plasticity.*

In addition, we have also provided a novel research paradigm for the investigation on how TFs regulate their responsive genes through chromatin interactions instead of direct binding at the gene body regions. ***Taken together, ERG is identified as a master transcription factor to manipulate plasticity in prostate cell lineage differentiation towards the pro-luminal programing through chromatin interactions.*** *Our findings can propose a novel working model for elucidating the detailed mechanisms for pursuing a fundamental and long-standing goal aimed at how prostate cancer cells actively maintain luminal lineage identities, as well as for providing the further supporting researches on the role of lineage plasticity in prostate cancer initiation*

6 OBSERVATIONS

Based upon the summary above we present several observations.

6.1 IN VITRO VS IN VIVO

There is always the issue of having vi vitro vs in vivo. Clearly the need to a tumor micro environment is an issue when trying to perform in vitro testing. Positive and negative environment interference is always a concern. Thus many of the Li et al observations fall withing that category.

6.2 MICE VS MEN

Murine models similarly have similar problems. We have argued before that despite their near universal application that moving to a human always presents substantial challenges.

6.3 THERAPEUTIC TARGETS

Can ERG thus present as a possible therapeutic target. As Blee et al have noted:

One potential therapeutic target is ERG, a transcription factor aberrantly up-regulated in PCa due to chromosomal rearrangements between androgen-regulated gene TMPRSS2 and ERG. Here we show that the most common PCa-associated truncated ERG T1–E4 (ERGΔ39), encoded by fusion between TMPRSS2 exon 1 and ERG exon 4, binds to bromodomain-1 (BD1) of bromodomain containing protein 4 (BRD4), a member of the bromodomain and extraterminal domain (BET) family. This interaction is partially abrogated by BET inhibitors JQ1 and iBET762. Meta-analysis of published ERG (T1–E4) and BRD4 chromatin immunoprecipitation-sequencing (ChIP-seq) data demonstrates overlap in a substantial portion of their binding sites. Gene expression profile analysis shows some ERG-BRD4 co-target genes are upregulated in CRPC compared to hormone-naïve counterparts.

We provide further evidence that ERG-mediated invasion of PCa cells was significantly enhanced by an acetylationmimicking mutation in ERG that augments the ERG-BRD4 interaction. Our findings reveal that PCa-associated ERG can interact and co-occupy with BRD4 in the genome, and suggest this druggable interaction is critical for ERG-mediated cell invasion and PCa progression.

A similar analysis has been done by Asangani et al (2014).

JQ1-treatment had a marked effect on ERG expression in VCaP cells and we found that the attenuation of DHT-induced ERG expression by JQ1 was due to de-recruitment of RNA PolII from ERG gene body and reduced binding of AR and BRD4 on the TMPRSS2 promoter/enhancer.

The efficient ERG downregulation by JQ1 has significant implication as TMPRSS2-ERG gene fusion product is the oncogenic driver in 50% of prostate cancers. *…*

We next determined the functional consequence of JQ1 treatment by measuring the expression levels of select ERG target genes. …

Further, we found that ERG was highly enriched on the known distal-enhancer of MYC that was reduced upon JQ1-treatment.

Likewise, ETV1 occupies the same distal-enhancer region in ETV1 fusion-positive LNCaP23. Knockdown of ERG or ETV1 along with AR led to MYC down-regulation, implicating MYC regulation by ETS proteins in fusion-positive prostate cancer cells (Extended Data Fig. 8c-e). … Lack of de-repression of MYC by JQ1 in this setting could be explained by the fact that both AR and ERG are absent from the MYC distal-enhancer leading to net loss of MYC expression. This data also suggests a mechanism by which CRPC patients become resistant to anti-androgen therapy by maintaining expression of the MYC oncogene.

Perhaps there may be many other such targets.

6.4 EPIGENETIC FACTORS

Epigenetic factors often drive malignant states and several are seen in PCa. As Baumgart and Haendler have noted:

Prostate cancer affects an increasing number of men worldwide and is a leading cause of cancer-associated deaths. Beside genetic mutations, many epigenetic alterations including DNA and histone modifications have been identified in clinical prostate tumor samples. They have been linked to aberrant activity of enzymes and reader proteins involved in these epigenetic processes, leading to the search for dedicated inhibitory compounds. In the wake of encouraging anti-tumor efficacy results in preclinical models, epigenetic modulators addressing different targets are now being tested in prostate cancer patients. In addition, the assessment of microRNAs as stratification biomarkers, and early clinical trials evaluating suppressor microRNAs as potential prostate cancer treatment are being discussed….

Inhibitors directed at the zinc-dependent HDAC family members have been around for many years, and their impact on the acetylation status of histone and non-histone proteins were described by numerous groups. Their efficacy was evidenced in several prostate tumor models. In vivo activity was, for instance, reported for the pan-HDAC inhibitors panobinostat and belinostat, and for the more selective inhibitors entinostat and mocetinostat . Panobinostat was also shown to block growth of castration-resistant models

Importantly, a stronger impact of HDAC inhibitors was observed in models harboring the ERG gene fusion, which is detected in about 50% of prostate tumors. Concerning NAD+–dependent HDACs, it was described that sirtuin 1 directly interacts with the AR to locally reduce histone acetylation and repress its activity…

The role of BET proteins, mainly BRD4, in prostate cancer has been reported by several groups. Inhibitors of BET bromodomains with various chemical scaffolds such as JQ1, OTX015/MK-8628, I-BET762 or ABVV-075 exhibit strong anti-proliferative effects in different tumor xenografts, including models that respond poorly to anti-androgens.

A reduction of the expression and binding of AR full-length and of a splice variant found in resistant tumors was reported. Another study shows that a model bearing an AR mutation responsible for enzalutamide resistance is still responsive to a combination treatment with JQ1.

Also, BRD4 interacts with ERG to control the expression of common target genes which are up-regulated in CRPC. BET bromodomain inhibitors such as JQ1 and I-BET762 can partially prevent this interaction, implying an additional mechanism by which they reduce prostate tumor growth.

6.5 STEM CELLS

The argument of the cancer cell or origin and/or the cancer stem cell, albeit different but equally compelling, has been an ongoing set of discussions[12]. We have looked at these issues in detail for the past decade with ever evolving answers. If ERG is such a master anything then does this become a target for identifying such a cell?

6.6 ERG EXPRESSION AND MEANING

ERG is a transcription factor. As such it assists in the transcription of other genes. If ERG is a "master transcription factor" or master anything one should have a full grasp of its functioning. Thus reasonable questions regarding ERG may be as follows:

1. What transcription factor assist in the expression of ERG

2. What promoter assists in the expression of ERG

3. What are the complete set of pathway elements involved in the ERG expression.

4. What are the complete set of pathway actions controlled by ERG

5. What are the extracellular influences on ERG expression including ligand actions as well as micro-environment actions.

6. Thus for TMPRSS2 fusion, we can ask the same set of questions regarding ERG and its functions in that context.

Some of these issues have been addressed and we will comment below, adding to what we have discussed internally.

[12] https://www.researchgate.net/publication/301222986_Prostate_Cancer_Stem_Cells and https://www.researchgate.net/publication/301542243_Cancer_Stem_Cells_and_Cancer_of_Origin_Redux

As Attard et al have noted:

Hormone-driven expression of the ERG oncogene after fusion with TMPRSS2 occurs in 30% to 70% of therapy-naive prostate cancers. Its relevance in castration-resistant prostate cancer (CRPC) remains controversial as ERG is not expressed in some TMPRSS2-ERG androgen-independent xenograft models. However, unlike these models, CRPC patients have an increasing prostate-specific antigen, indicating active androgen receptor signaling. Here, we collected blood every month from 89 patients (54 chemotherapy-naive patients and 35 docetaxel-treated patients) treated in phase I/phase II clinical trials of an orally available, highly specific CYP17 inhibitor, abiraterone acetate, that ablates the synthesis of androgens and estrogens that drive TMPRSS2-ERG fusions.

We isolated circulating tumor cells (CTC) by anti–epithelial cell adhesion molecule immunomagnetic selection followed by cytokeratin and CD45 immunofluorescence and 4¶,6-diamidino-2-phenylindole staining. We used multicolor fluorescence in situ hybridization to show that CRPC CTCs, metastases, and prostate tissue invariably had the same ERG gene status as therapy-naive tumors (n = 31). We then used quantitative reverse transcription–PCRto show that ERG expression was maintained in CRPC. We also observed homogeneity in ERG gene rearrangement status in CTCs (n = 48) in contrast to significant heterogeneity of AR copy number gain and PTEN loss, suggesting that rearrangement of ERG may be an earlier event in prostate carcinogenesis.

We finally report a significant association between ERG rearrangements in therapy-naive tumors, CRPCs, and CTCs and magnitude of prostate-specific antigen decline (P = 0.007) in CRPC patients treated with abiraterone acetate. These data confirm that CTCs are malignant in origin and indicate that hormone-regulated expression of ERG persists in CRPC.

As Babu and Fullwood have noted:

Prostate cancer is the most common cancer in men older than 50 years. Investigations into the molecular basis of prostate cancer have identified several candidate drivers and genetic alterations. Of these, the TMPRSS2-ERG (T2E) fusion gene arising from genetic rearrangement leading to fusion of the TMPRSS2 gene (encoding transmembrane protease serine 2) and the ERG transcription factor gene (ETS-related gene) has been a central focus, as it was found to be associated with nearly half of prostate cancer cases.

However, how the T2E fusion gene drives the development of prostate cancer was unclear. In a recent report, Mathieu Lupien and colleagues used a variety of genomic profiling approaches on patient samples to show that altered chromatin states lead to enhanced ERG transcription in T2E-positive prostate cancer. The modified chromatin landscape in conjunction with expanded transcriptional activity leads to upregulation of other target genes involved in prostate development and cancer …

ERG overexpression leads to the development of super-enhancers that further drive the cell toward oncogenesis.

It is possible that abolishing this association through the disruption of DNA loops could lead to amelioration of disease pathology. This study furthers understanding of the biology of prostate cancer and has implications for the development of new precision therapies targeting T2E-positive prostate tumors

As Yu et al had noted:

In the present study, we systematically mapped the genomic landscape of AR, ERG, FoxA1, and RNA PolII, along with eight critical histone marks in multiple prostate cancer cell lines as well as in one prostate tumor specimen. These studies not only reveal important biological findings regarding the mechanisms of TMPRSS2-ERG gene fusions in prostate cancer, but they also provide a compendium of 57 genome-wide ChIP-Seq experiments and a large set of paired microarray expression profiling data that will be useful for the investigation of biological mechanisms of cancer and steroid hormone receptor signaling.

By analyzing these genome-wide maps, we provide a working model of how TMPRSS2-ERG gene fusions regulate prostate cancer progression. In the context of an androgen-regulated gene fusion such as TMPRSS2-ERG, this fusion product can attenuate androgen signaling by multiple, cooperative mechanisms including direct inhibition of AR expression and attenuation of AR signaling at gene-specific loci (Figure 7H). Furthermore, our study reveals an additional pathway of ERG in perturbing cell differentiation through the Polycomb group proteins. Enrichment of H3K27me3-marked genes silenced in ESCs and aggressive tumors was first apparent by MCM analysis of AR-occupied genes in prostate cancer, linking both AR and ERG to repressive epigenetic signatures (Figure 1C).

This was further substantiated in prostate cancer tissues harboring ERG gene fusions being distinguishable by H3K27me3-containing and/or Polycomb-occupied genes.

ERG was found to be a direct activator of EZH2 and the level of EZH2 expression was associated with the ERG status in a cohort of prostate tumors.

Thus, TMPRSS2-ERG plays a central role as a ‘‘malignant regulatory switch’’ that shuts down androgen signaling, inhibiting normal prostate differentiation and turning on EZH2 expression, which induces an ESC-like dedifferentiation program.

Because TMPRSS2-ERG gene fusions are androgen-responsive, they were thought to merely represent one of many mutated pathways emanating from AR signaling.

Our results, however, suggest that TMPRSS2-ERG plays a much more fundamental role. As an early-onset genetic lesion, TMPRSS2-ERG gene fusion may provide a mechanism for AR overexpression and mutation in advanced prostate cancer.

Antiandrogen treatments such as bicalutamide or flutamide are currently being used to treat advanced disease. Unfortunately, patients treated with these AR antagonists often develop recurrent disease that is resistant to this therapy. Tumors from men with castration-resistant metastatic prostate cancer (CRMPC) often overexpress AR through multiple mechanisms including AR amplification.

Repression of AR by TMPRSS2-ERG may provide a malignant selection pressure contributing to recurrent tumors with AR amplification. This is supported by our observation of a negative correlation (r = 0.35, p = 0.0014) between AR and ERG expression in localized prostate tumors, but a positive correlation (r = 0.30, p = 0.058) in metastatic prostate cancers. Further, whereas AR amplification on its own is not sufficient to induce hyperplastic lesions, overexpression of a CMV-promoter driven AR (thus not susceptible to ERG repression, mimicking a hormone-refractory state with AR amplification), together with forced ERG overexpression, has recently been shown to promote the development of a more poorly differentiated, invasive adenocarcinoma.

This may also suggest that therapies targeting AR may not produce a durable response in prostate cancer patients when the underlying mutation may in fact be TMPRSS2-ERG. Paradoxically, therapies employing high-dose testosterone may have a beneficial effect transiently by favoring a normal differentiation state. Consistent with this, preclinical models suggest that high doses of exogenous testosterone inhibit prostate cancer growth while lows levels of testosterone promote tumor growth. Recently, high doses of exogenous testosterone have been shown to be safe in patients with CRMPC. Bicalutamide and flutamide exhibit a partial agonistic effect that may also promote normal prostate differentiation, which is eventually overcome by TMPRSS2-ERG expression and consequent resistant disease.

Taken together, our findings provide a working model in which TMPRSS2-ERG plays a critical role in cancer progression by disrupting the AR lineage-specific differentiation program of the prostate and favoring EZH2-mediated cellular dedifferentiation. In addition, by inhibiting AR signaling, TMPRSS2-ERG may exert a selective pressure for the development of prostate cancer that is resistance to hormone-deprivation therapies. Furthermore, our study provides a compendium of 57 ChIP-Seq experiments of key transcription factors and histone modifications in prostate cancer, which will be invaluable for prostate cancer and steroid hormone research

6.7 DETECTION

There is a significant interest in detection by non-invasive means, namely blood and urine testing. The use of an ERG related target has been of interest. From O'Reilly et al:

Some other prognostic urine indicators of aggressive PCa have already been described. For example, the Mi Prostate Score (MiPS), which incorporates the prostate-specific TMPRSS2-ERG fusion and PCA3 transcripts in urine, in conjunction with serum PSA, delivers AUCs of 0.75 and 0.78 for detecting all PCa and high-grade PCa, respectively. However, the prognostic value of this combination has been questioned by others.

Combining expression of three genes (HOXC6, TRD1, and DLX1) functionally implicated in PCa with serum PSA detected clinically significant disease (Gleason score greater than or equal to 7 on biopsy), with an AUC of 0.81.

Thus detecting ERG may have some merit. Just what density of ERG is an open question. Also do we detect blood or urine. Or is it necessary to do an invasive test?

6.8 MASTER REGULATORS

The open issue in this Note is the assertion that ERG is a "Master Transcription Factor". Master Factors of various types have been asserted by many researchers. Califano's group at Columbia have examine many such Master Factors. As Shen et al have noted:

Critically, these methods lack cell-context specificity and are limited to assessing only direct, high-affinity binding compounds, thus missing small-molecule compounds that may indirectly modulate the activity of a target protein, as is the case for ibrutinib. These compounds cannot be assessed by QSAR, because they do not represent high-affinity ligands of the target protein of interest but rather of one of its major context-specific up-stream regulators. In addition, these methods are not effective for protein families that lack specific binding pockets, such as transcription factors (TFs) even though these comprise many of the best established tumor dependencies. Indeed, TFs such as ESR1, NOTCH1, MYC, GATA3, and ERG, among many others, are frequently aberrantly activated in cancer. In addition, many TFs have been recently elucidated as ***Master Regulators*** *of tumor cell state, which are organized in highly interconnected modules or tumor checkpoints, including key synthetic lethal combinations, such as STAT3, CEBPB, and CEBPD in mesenchymal glioblastoma or CENPF and FOXM1 in malignant prostate carcinoma*

Due to their direct effect on the cell transcriptional response, TRs are at the center of the machinery that integrates exogenous and endogenous signals to control physiologic and pathologic cell states. We have shown that master regulator (MR) TRs responsible for pathological transitions, can be systematically elucidated by differential activity analysis but not by differential expression. These MR TRs constitute key non-oncogene tumor dependencies, eliciting either essentiality or synthetic lethality in vitro and in vivo. Unfortunately, TRs are considered to be "undruggable" targets because the DNA-interacting surface is highly charged resulting in unfavorable drug target properties

Li et al have asserted ERG as a MTF. It may be but given the data thus far one can seek additional clarification. However as a putative MTF it does present an interesting target for therapeutics.

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