Prostate Cancer Metastasis: Some Simple Cases

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In previous work we have developed some generic models for cancer metastasis. In this paper we attempt to obtain actual experimental data and apply it to the model demonstrating that it is workable. We also examine various elements of the model in the light of recent results demonstrating metastatic PCa gene expression states.

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1 INTRODUCTION

Cancer metastasis has generally considered that cancer is clonal, namely it starts with a single cell and that cell starts a process that laves its local environment and through a process of continual change manages to metastasize throughout the body. We examine a recent paper by Gundem et al which examines the metastatic behavior of prostate cancer and as a result of GWAS they show that it can be poly clonal and continually changing.

We the return to a paper we prepared well over a year ago regarding Cancer Dynamics and show that in that paper we had not only anticipated this but more. Although that paper does not yet treat epigenetic factors, nor does Gundem et al, it can be readily modified to do so.

The results have some significant consequences. Mostly is the treatment of such cancers. Namely if we have polyclonal metastatic propagation then pathway methods may have to be multifaceted, namely dealing with the multiplicity of differing pathway anomalies.

1.1 RECENT RESEARCH

In a recent paper by Gundem et al the authors describe an analysis they have performed on metastatic prostate cancer cells in a group of patients. Their general conclusions seem to be two fold; (i) that there are certain metastases that are polyclonal, namely there are multiple cells initiating the process, (ii) that the progression of the metastases is complex with ever increasing changes in genetic expression.

Gundem et al state:

By plotting the cancer cell fractions of mutations from pairs of samples, we determined the clonal relationship between the constituent subclones and found evidence for polyclonal seeding of metastases,

This is a powerful observation. Their approach was in simple terms to do genome wide analysis and doing so over a set of metastatic locations. Then using a clustering method they could determine with reasonable accuracy the clonal and polyclonal results as well as the progression. Specifically:

Using whole-genome sequencing, we characterized multiple metastases arising from prostate tumours in ten patients. Integrated analyses of subclonal architecture revealed the patterns of metastatic spread in unprecedented detail. Metastasis-to-metastasis spread was found to be common, either through de novo monoclonal seeding of daughter metastases or, in five cases, through the transfer of multiple tumour clones between metastatic sites.

Lesions affecting tumour suppressor genes usually occur as single events, whereas mutations in genes involved in androgen receptor signalling commonly involve multiple, convergent events in different metastases. Our results elucidate in detail the complex patterns of metastatic spread

and further our understanding of the development of resistance to androgen-deprivation therapy in prostate cancer ... We identified a set of high-confidence substitutions, insertions/deletions, genomic rearrangements and copy number changes present in each tumour sample….

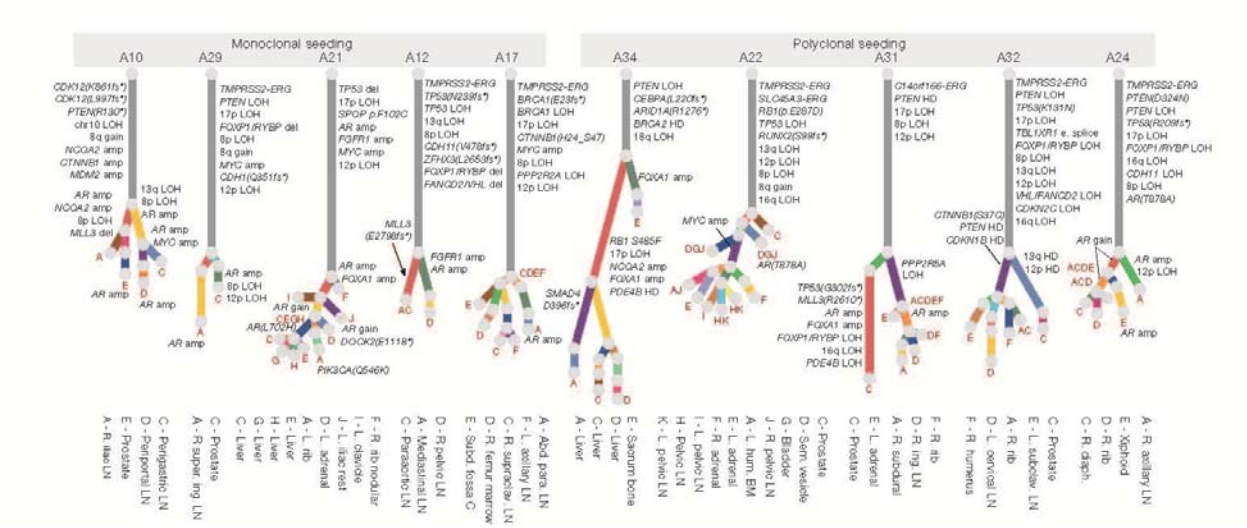
They conclude as follows:

Our analyses allow us to view with unprecedented clarity the genomic evolution of metastatic prostate cancer, from initial tumorigenesis through the acquisition of metastatic potential to the development of castration resistance. A picture emerges of a diaspora of tumour cells, sharing a common heritage, spreading from one site to another, while retaining the genetic imprint of their ancestors. After a long period of development before the most recent complete selective sweep, metastasis usually occurs in the form of spread between distant sites, rather than as separate waves of invasion directly from the primary tumour.

This observation supports the 'seed and soil' hypothesis in which rare subclones develop metastatic potential within the primary tumour, rather than the theory that metastatic potential is a property of the primary tumour as a whole. Transit of cells from one host site to another is relatively common, either as monoclonal metastasis-to-metastasis seeding or as polyclonal seeding. Clonal diversification occurs within the constraining necessity to bypass ADT, driving distinct subclones towards a convergent path of therapeutic resistance. However, the resulting resistant subclones are not constrained to a single host site. Rather, a picture emerges of multiple related tumour clones competing for dominance across the entirety of the host.

The challenge in the above analysis is to note as we had in Cancer Dynamics that as the genetic profile of the cancer cells change, there is a survival of the fittest occurring, namely a certain cell tries to dominate, and there is also the issue of stem cells and stem cell control and proliferation. The issue is one of understanding just what constitutes metastatic growth. Clearly the cells are in a steady state of genetic change, altering in a survival based manner to dominate.

Figures 3 and 4 of the paper are the most significant. In Figure 3 we see depicted the evolving changes in gene structure in clonal and polyclonal mets. In Figure 4 we see the same in a Nuclear Medicine scan showing the mets. We show that Figure from Gundem et al below since it is of such significance.



The above shows the mutations or gene expression alterations and as they progress. This is a complex but quite important description of the process. (NOTE: The above is Figure 3 as modified from Gundem et al, Nature, 2015).What this shows is the gene expression variants by location in a metastatic environment. What are of interest are the various changes by gene and the sequence of the changes and the progression by location. One may use this as a template for much of what we have been discussing before as well as in this analysis.

As Shen states in a Nature commentary on the Gundem et al paper:

Next-generation DNA sequencing technologies have made it apparent that primary tumours are not clonal (consisting of a single population of genetically identical cells). Instead, they are composed of subclones, subpopulations of genetically identical cells that can be distinguished from other subclones by the mutations they harbour. Such subclones compete for dominance during cancer progression, and drug treatment can lead to formerly minor tumour subclones becoming dominant if they are resistant to treatment.

Thus, clonal evolution shapes the properties of tumours and can explain their plasticity in response to therapy. Until now, however, clonal evolution has not been explored in detail in the context of metastasis…..Taken together, the current studies might explain why, given the prevalence of circulating tumour cells in patients with solid tumours, successful metastasis is relatively rare — metastasis may be facilitated by seeding by cell clusters containing cooperating clones with distinct properties.

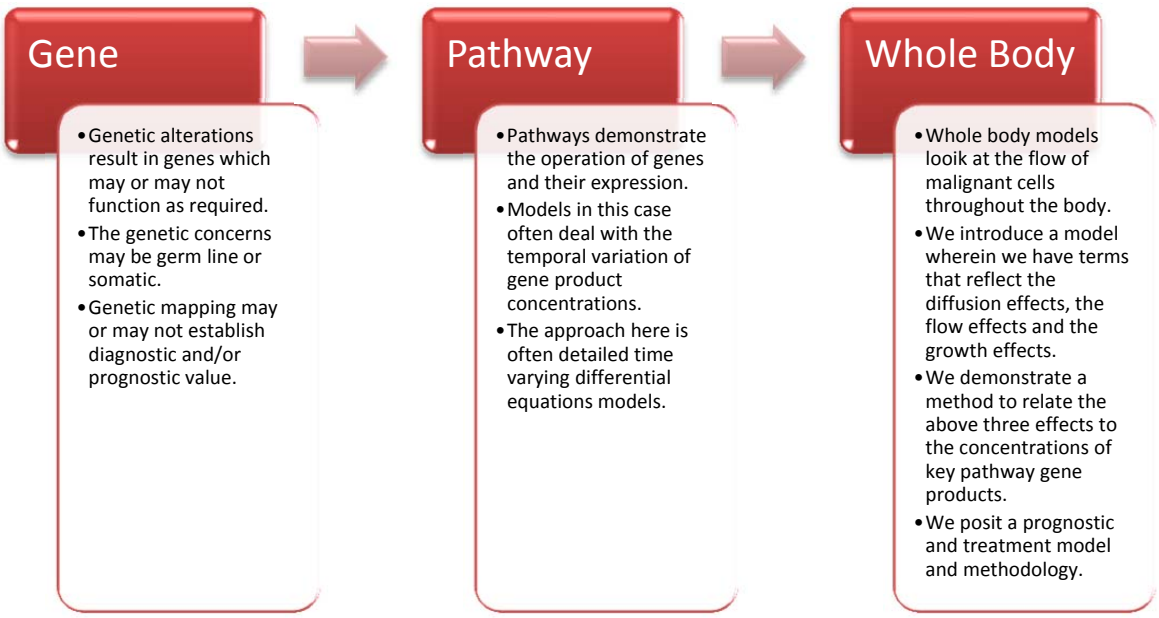
If so, it is attractive to speculate that disseminated single cells could remain dormant until reawakened by interaction with a cooperative metastatic cell arriving at the same secondary site. Such a model has the potential to revise our conception of the properties of tumour-initiating cells, as well the metastatic niche, and may have implications for therapeutic strategies. For example, understanding the signalling pathways that mediate such clonal cooperativity may lead to effective therapies using drugs that target these pathways.

The signaling pathway issue is a complex one especially since we know that suppressing one pathway may excite another. The problem will be targeting all of the cells.

1.2 PREVIOUS WORK

We have considered this before when we wrote a detailed paper in 2013 on Cancer Dynamics. In our analysis we examined a set of continually changing cancer cells, and we further assumed that any cell may have changed to a cancer cell. We then further assumed a diffusion/flow model for the propagation of those cells and at the same time assumed a continual process of genetic change. We also assumed that we could find an organ specific environment which may be most favorable to growth via ligand/receptor combinations. Finally we also assumed that cell to cell communications could facilitate the process. We did not consider at that time any epigenetic factors.

Namely when considering cancer propagation we must consider the genes, the pathways and the whole body. It is a complex process which we had developed in the referred to paper.



The equations for the propagation over space and time for a specific type of cell containing a specific genetic makeup has been shown below. Here n(x,t) is the concentration or density of a specific cell type, let us assume a malignant prostate cancer cell, and with a specific genetic profile. If we examine the Gundem paper we see that this is what they are looking at from the perspective of a GWAS study of metastatic PCa. However we have already developed a model and further we had developed an identification process to provide the drivers in the model itself.

Note below that the general equation is a diffusion plus flow model, diffusion due to evolving concentrations and flow due to movement within the body itself such blood flow dissemination.

$$\frac{\partial \overline{n(x,t)}}{\partial t} = \tilde{L}\overline{n(x,t)} + \Lambda \overline{n(x,t)}$$

$$\tilde{L} = \begin{bmatrix} L_1 - \lambda_{11}, 0, \ldots\ldots\ldots, 0 \\ \ldots\ldots \\ 0, 0, \ldots\ldots\ldots 0, L_n - \lambda_{nn} \end{bmatrix}$$

$$L_k = a_k \frac{\partial^2(\ldots)}{\partial x^2} + b_k \frac{\partial(\ldots)}{\partial x} + c_k(\ldots)$$

$$\Lambda = \begin{bmatrix} 0, \lambda_{12}, \ldots\ldots, \lambda_{1n} \\ \ldots\ldots \\ \lambda_{n1}, \ldots\ldots\ldots, 0 \end{bmatrix}$$

The L values are operators and the others are constants determined in the paper. Our model then allows for polyclonal development and moreover a complex cell to cell growth stimulus as well.

1.3 OBSERVATIONS

Now Cancer UK comments on this work as follows[1]:

The team has already revealed a huge amount of genetic diversity between cancer cells taken from different sites within each man's prostate…this new study shows that, despite the diversity, prostate cancer cells that break free from the tumour and spread share common genetic faults unique to the individual patient.

Study author … said: "We gained a much broader view of prostate cancer by studying both the original cancer and the cells that had spread to other parts of the body in these men. And we found that all of the cells that had broken free shared a common ancestor cell in the prostate. The common faults we found in each man could potentially offer new targets for treatment. But we found that, once cancer cells have spread, they continue to evolve genetically, so choosing the most effective treatments will remain a key challenge."

[1] http://www.cancerresearchuk.org/about-us/cancer-news/press-release/2015-04-01-scientists-drill-down-to-genetic-root-of-prostate-tumour-development also see http://www.sciencedaily.com/releases/2015/04/150401161514.htm and http://www.sciencedaily.com/releases/2015/04/150402114659.htm and http://scienceblog.cancerresearchuk.org/2015/04/04/news-digest-prostate-cancer-family-tree-pineapples-walnuts-and-more/

"The diversity we've found suggests multiple biopsies might be needed to identify the 'trunk' of the cancer's tree of mutations – we need treatments that target these core weaknesses to destroy all cancer cells in a clean sweep, rather than trimming the branches. We must also study more patients to learn how to apply these findings to develop more personalised treatments for people with the disease."

"In the phylogenetic trees that our data have produced, we see that most of the oncogenic mutations are shared clonally by all the tumour sites in each patient. This common genetic heritage is a potential achilles heel of the metastases, however, many of these shared mutations are in tumour suppressor genes and our approach to therapeutically targeting these needs to be prioritised.

"It takes a while before a tumour develops the ability to metastasise but once it does the patient's prognosis changes significantly. We have to zoom in on this crucial junction and gather more data on the impact different therapies have on prostate cancer's evolution and spread."

Moreover there are many more concerns. For example:

1. Epigenetic Factors: The analysis does not appear to deal with the epigenetic factor such as methylation, miRNAs, lncRNAs and the like. We clearly know that they also have significant impact.

2. Stem Cell Issues: There is also the issue of the stem cell. Is there such a factor included in or includable in this analysis?

3. Pathway Modifying Therapeutics: As discussed by one of the commentators the therapeutic implications are evident but in our opinion not at all clear.

4. Prognosis Analysis: Here we have a significant concern. Many prognostic tests have been developed. However if we examine for one gene profile are we missing many others due to poor sampling. Namely one type of polyclonal cells may be in the profile match but another may not. How, then does this observation impact the many PCa prognostic profiles out today?

1.4 SOME GENES

From Gundem (as modified) we have the following Table which presents the genes whose expression is modified in PCa. They use four sources and indicate for each source the inclusion of the specific gene. We have ranked them from all 4 to only a single one to give some semblance of significance.

Gene	Cancer Gene Census	Cancer 5000 Any Cancer (q<0.05) (92)	Grasso et al 2012	Barbieri et al. 2012	Total
APC	1	1	1	1	4
ARID1A	1	1	1	1	4

Gene	Cancer Gene Census	Cancer 5000 Any Cancer (q<0.05) (92)	Grasso et al 2012	Barbieri et al. 2012	Total
BAP1	1	1	1	1	4
CDH1	1	1	1	1	4
CDKN1B	1	1	1	1	4
ERBB2	1	1	1	1	4
FOXA1	1	1	1	1	4
KDM6A	1	1	1	1	4
MLL	1	1	1	1	4
MLL2	1	1	1	1	4
MLL3	1	1	1	1	4
PBRM1	1	1	1	1	4
PIK3R1	1	1	1	1	4
PTEN	1	1	1	1	4
SETD2	1	1	1	1	4
SF3B1	1	1	1	1	4
SMAD2	1	1	1	1	4
SPOP	1	1	1	1	4
TBX3	1	1	1	1	4
TP53	1	1	1	1	4
XPO1	1	1	1	1	4
ASXL1	1	1	1	0	3
ASXL2	1	0	1	1	3
ATM	0	1	1	1	3
BCOR	1	1	0	1	3
CARD11	1	1	1	0	3
CASP8	1	1	0	1	3
CDK12	1	1	1	0	3
CDK4	1	1	1	0	3
CTNNB1	1	1	1	0	3
EGFR	1	1	0	1	3
EP300	1	1	0	1	3
FLT3	1	1	1	0	3
HIST1H3B	1	1	1	0	3
HSP90AB1	1	1	1	0	3
IDH1	1	1	0	1	3
KDM5C	1	1	0	1	3
KRAS	1	1	1	0	3
MAP2K1	1	1	1	0	3

Gene	Cancer Gene Census	Cancer 5000 Any Cancer (q<0.05) (92)	Grasso et al 2012	Barbieri et al. 2012	Total
MET	1	1	1	0	3
NF1	1	1	1	0	3
NOTCH1	1	1	1	0	3
PHF6	1	1	1	0	3
PIK3CA	1	1	0	1	3
RB1	1	1	1	0	3
TET2	1	1	1	0	3
AKT1	1	1	0	0	2
ALK	1	1	0	0	2
ARID2	1	1	0	0	2
BRAF	1	1	0	0	2
BRCA1	1	1	0	0	2
CCND1	1	1	0	0	2
CD79B	1	1	0	0	2
CDKN2A	1	1	0	0	2
CEBPA	1	1	0	0	2
CHD1	0	0	1	1	2
CREBBP	1	1	0	0	2
DDX3X	0	1	1	0	2
DNMT3A	1	1	0	0	2
ERCC2	1	1	0	0	2
EZH2	1	1	0	0	2
FBXW7	1	1	0	0	2
FGFR2	1	1	0	0	2
FGFR3	1	1	0	0	2
GATA3	1	1	0	0	2
HRAS	1	1	0	0	2
IDH2	1	1	0	0	2
KEAP1	1	1	0	0	2
KIT	1	1	0	0	2
MAP2K4	1	1	0	0	2
MED12	1	1	0	0	2
MTOR	0	1	0	1	2
MYD88	1	1	0	0	2
NCOA2	1	0		1	2
NFE2L2	1	1	0	0	2
NPM1	1	1	0	0	2

Gene	Cancer Gene Census	Cancer 5000 Any Cancer (q<0.05) (92)	Grasso et al 2012	Barbieri et al. 2012	Total
NRAS	1	1	0	0	2
PPP2R1A	0	1	1	0	2
PRDM1	1	1	0	0	2
PTPN11	1	1	0	0	2
RAC1	1	1	0	0	2
RUNX1	1	1	0	0	2
SMAD4	1	1	0	0	2
SMARCA4	1	1	0	0	2
SMARCB1	1	1	0	0	2
STK11	1	1	0	0	2
TGFBR2	1	1	0	0	2
U2AF1	1	1	0	0	2
VHL	1	1	0	0	2
WT1	1	1	0	0	2
ZFHX3	0	0	1	1	2
CTCF	0	1	0	0	1
ERBB3	0	1	0	0	1
MAP3K1	0	1	0	0	1
NCOR1	0	0		1	1
PDE4B	0	0	1	0	1
PPM1D	0	1	0	0	1

1.5 HGPIN AGAIN

Before discussing metastatic behavior, let us recount HGPIN. As we have discussed previously, HGPIN is a complex process which many believe is a predictor of PCa. On the other hand there is clinical evidence that HGPIN may disappear. Thus one asks if HGPIN may be an inflammatory reaction which can be abated or that in the biopsy process of determining HGPIN a stem cell progenitor could have been removed in the process.

Thus one of the questions we can ask to attempt to validate the model is to focus on HGPIN and its known genetic properties in HGPIN and PCa. Specifically it is the loss of expression of GSTP1 via methylation that appears to be a driver for HGPIN and possibly PCa. From Inoue et al[2]:

[2] http://cancerres.aacrjournals.org/content/68/9_Supplement/4281.short

GSTP1 has been proposed to be a caretaker gene, protecting cells against genome damage. It has been shown that the GSTP1 protein is absent in prostate cancer (CaP) and high grade prostatic intraepithelial neoplasia (HGPIN). The absence of GSTP1 protein in CaP and HGPIN is related to hypermethylation of the GSTP1 CpG island.

Previously we reported that while normal epithelium from cancer patients does not contain methylated GSTP1, 6% of atrophy lesions, 68.8% of HGPIN lesions and 90.9% of CaP lesions harbor methylated GSTP1 alleles. Moreover, density of methylation, as well as presence of methylation was likely to be associated with prostate carcinogenesis. However, the pattern of methylation of individual alleles has not been investigated previously. We used bisulfite sequencing to investigate methylation status of 39 CpG sites within the GSTP1 promoter….

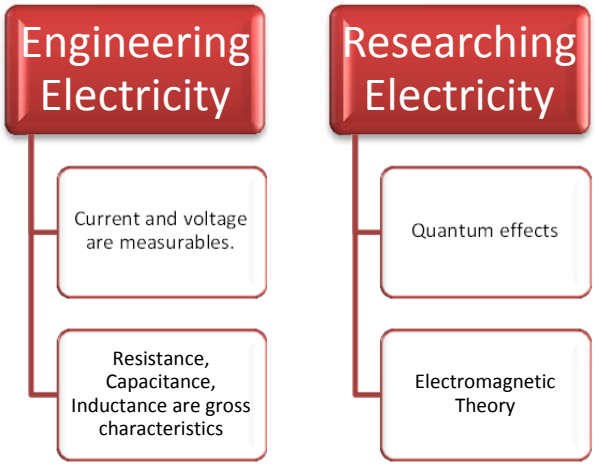
The results support the hypothesis that methylation of individual CpG sites in the island gradually increases during progression of the neoplastic process. Since HGPIN lesions were clearly intermediate between normal and cancer, these results further support the concept that HGPIN lesions are indeed part of the stepwise molecular progression in prostate carcinogenesis.

Thus if we have methylation of the CpG islands and a suppression of GSTP1 then it should be continued as the cells proliferate. Here we have a simple first target for validating the model. A single gene suppression and based upon that we should be able to ascertain the result by multiple biopsies. For example we should understand the rate of proliferation and if HGPIN is detected on a first biopsy then we should see even more on a second and so on. If not, then we should ask why not?

1.6 OVERVIEW

We briefly give an overview of this report.

1. Sections on the model address first the model, second some simple assumptions we can make and third we attempt to present a simple solution. The problem we find is that the constants are not available. Again our approach is akin to what was done in Electricity where on the one hand there is a complex set of research issues from materials and quantum effects to complex electromagnetic theory issues. Yet one can solve a great many engineering problems know a few gross variable.



2. Intra and Extra vasation is considered as a flow process. This is a difficult extension since this process may be quite complex. The cells generally do not diffuse or grow in the blood stream but may exhibit some behavior in the lymphatic system, and we generally disregard those effects. We examine the process to lend some substance top the model.

3. We then proceed to consider some recent factors resulting in PCa. The problem we have is that almost daily we see some "extraordinary" result that must be considered. However many of these are reiterations of past work or merely are observations without any structure behind them. Epigenetic factors play a significant role in PCa whether they are methylation or miRNAs. Thus this examination provides some footing in that area.

4. We examine controlling factors and their suppression by other genes expressions. Growth factors can be controlled by other genes. Thus when modelling we must balance extra cellular and intra cellular factors. We examine some recent work here.

5. There is a never ending flow on gene profiles and recent ones being called "Rosetta Stones". We examine a recent on as an example and demonstrate that a great deal is a rehashing of well know factors and other elements no actionable.

6. We then use some of the data gathered to examine how one may go about estimating the constants, then examining what these estimates can be used for, and perform a specific analysis related to HGPIN. This is our first application of the model for a clinical application.

Our overall objective in this discussion is to examine some of the fundamental biological issues in the context of the gross model for cancer propagation. The questions are posed more from an engineering perspective of models for gross analysis rather than fundamental scientific questions. The primary examples are:

1. Growth Rates: Let us assume that we are examining HGPIN. We know from various studies that GSTP1 is down regulated by methylation, thus allowing proliferation of cells. Now if we were to know the proliferation rate resulting from this down regulation we could determine statistically what the prostate volume of such cells would be and could then more effectively

predict on second biopsies what to expect. Likewise using data from Gundem et al on cell mutation changes we could predict the more aggressive transitions in PCa.

2. Flow Rates: We examine the intravasation and extravasation process. This ate the biological level is highly complex and presents many yet to be determined factors. However in a gross physical model the concept of a flow rate can still hold. We discuss this at length.

3. Diffusion Rates: We have been examining such factors. These result from loss of adhesion and we examine some of those issues herein. We have yet to find adequate data to determine such factors in the literature. However experimental determination seems quite readily obtainable.

4. Mutations: Here we rely upon the work of Gundem et al. As we demonstrated above there are multiple changes in gene expression and they have been detailed by the authors. These are not inclusive but exemplary. The methodology also presents a methodology to determine the rates however.

2 A SIMPLE CASE

We will now consider a simple case. We will not assume either a stem cell or a single cell source. We will assume that a progression of genetic changes occur, each change will result in a progressive step forward. We will use the mathematical model we have previously developed to determine the movement of the malignancy. We will then use this model to demonstrate how one can estimate the variables involved, namely demonstrate the observability of the system.

Now consider the following example:

1. In the initial state we have a benign prostate with basal and luminal cells. The net change in total cells per unit volume at any period of time is zero. Furthermore the change in the genetic profile of any cell, either through proliferation of just as a normal process is zero. This is the ground state of the system. The somatic cells are fundamentally benign. We identify the cells here by the notation $n_1(x,t)$. In our model this means that the change of n is zero. Nothing changes.

2. Now we assume that some cell has been affected by some process that results in an increase proliferation of cells. The cells are still kept in place by such factors as E cadherin and talin (Tal-1) but the number of cells is increasing. Thus we may have had a change in GSTP1 which is exhibited in this proliferation. The cells proliferating may be basal or luminal and this represents the state above the initial ground state. As above we can now identify these cells as $n_2(x,t)$. In this case and in our model this means that the cells do not move or diffuse but that they proliferate.

In the above two cases we assume that the cells are in Region 1, in this case they are in the prostate. In the first case we have benign prostate tissue and in case 2 we have possibly HGPIN. The second case is proliferation into the glandular lacuna.

3. Now another mutation occurs, say suppressing E cadherin. This means that the cells, still proliferating, but now can diffuse as well. We have two factors at play, a proliferation factor and now a diffusion factor. There is not yet any flow factor and the cells remain in the same general region but are on the move solely because of density. The cells can diffuse but cannot pass the blood barrier. We identify these cells by $n_3(x,t)$.

4. Now we have cells starting to abut the blood stream. They cannot yet cross it since that requires a flow mechanism change in the cell surface receptors.

5. Finally the cells flow out of the blood stream and land in various organs. In order for them to proliferate again there must be both a genetic change and the correct cellular environment to excite cell receptors and in turn cell pathways. This is where PTEN may be suppressed.

The general equation for our simple case is:

$$\frac{\partial n_i(x,t)}{\partial t} = a_i \frac{\partial^2 n_i(x,t)}{\partial x^2} + b_i \frac{\partial n_i(x,t)}{\partial x} + c_i n_i(x,t) - p_{i,i} n_i(x,t) + p_{i,i-1} n_{i-1}(x,t)$$

where

$$p_{i,i} = 1 - p_{i,i-1}$$

here.

We now have to write these for the five cases. First note the general variable we have:

Region	Variable	Transition Probability	c; Proliferation	a: Diffusion	b: Flow
1: Prostate	X1	0	0	0	0
2: Prostate	X2	p21= requires change to GSTP1 expression	c2	0	0
3: Prostate	X3	p32= requires change to cadherin expression.	0	a3	0
4: Blood	X4	p43= requires an as yet known expression	0	0	b
5: Bone	X5	p54= require loss of PTEN expression.	c5	a5	0

This then yields:

$$\frac{\partial n_1(x,t)}{\partial t} = c_1 n_1(x,t) - p_{1,1} n_1(x.t) = 0$$

$$\frac{\partial n_2(x,t)}{\partial t} = c_2 n_2(x,t) + p_{2,1} n_1(x,t) - p_{2,2} n_2(x.t)$$

$$\frac{\partial n_3(x,t)}{\partial t} = a_3 \frac{\partial^2 n_3(x,t}{\partial x^2} + p_{3,2} n_2(x,t) - p_{3,3} n_3(x.t)$$

$$\frac{\partial n_4(x,t)}{\partial t} = b_4 \frac{\partial n_4(x,t)}{\partial x} + p_{4,3} n_3(x,t) - p_{4,41} n_4(x.t)$$

$$\frac{\partial n_5(x,t)}{\partial t} = a_5 \frac{\partial^2 n_5(x,t)}{\partial x^2} + c_5 n_5(x,t) + p_{5,4} n_4(x,t) - p_{5,5} n_5(x.t)$$

This simple model describes the metastatic proliferation of prostate cancer through the prostate, blood stream and bone. Our problem is not twofold:

1. How do we determine the constants?

2. With the constants, how well does this describe real PCa?

A third question may also be posed. Namely:

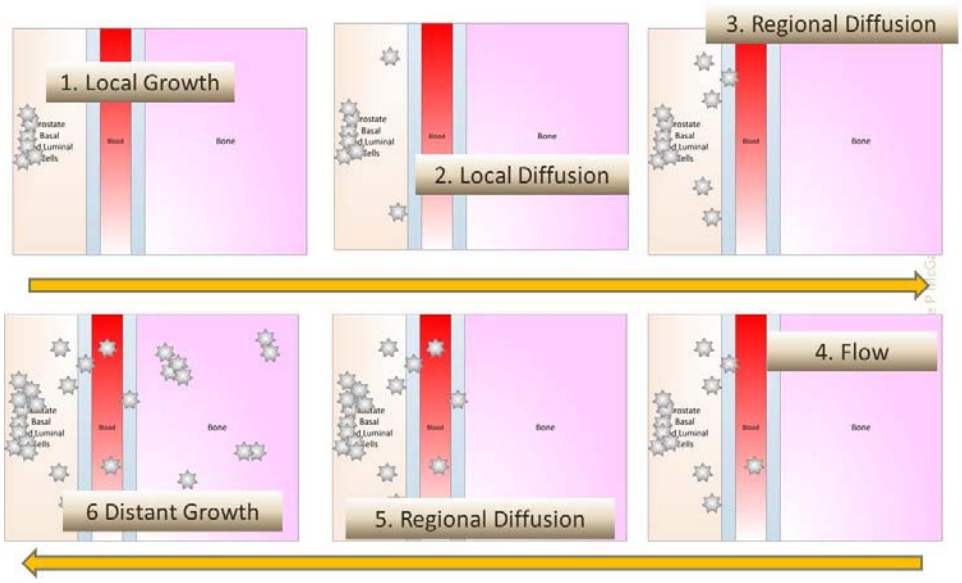
3. Having the model, having the constants, having the results which comply with reality; what can we do to reduce or eliminate the metastatic behavior? This is the controllability problem.

Now there are two observations about the constants.

1. The diffusion, flow and growth are somewhat constant.

2. The transition probabilities depend on expression changes and thus may change with time. For example if inflammation is a driver to GSTP1 suppression than this number may be a function of inflammation rate and time.

The Figure below depicts some of these in graphic form.



As shown above we proceed across several regions of growth.

3 SOME SIMPLE SOLUTIONS

Now we consider some simple solutions. These are linear equations and it should be clear that there is no steady state. Thus we examine dynamic solutions.

1. Region 1: In Region 1 we have merely zero or stable growth. Namely we have the original benign cell.

2. Region 2: In this region we have only a proliferation effect. The cells start to proliferate solely in the region. Thus we have a profile as follows:

$$n_2(x,t) = K_2 \exp(\lambda_2 t)$$
$$t > 0$$
$$x_{2,0} < x < x_{2,End}$$

3. Region 3: Now the cell loses its regional stability and begins to diffuse. It does the following in this region:

$$n_3(x,t) = K_3 \exp(-\frac{1}{2\sigma t} x^2)$$
$$t > 0$$
$$x_{3,0} < x < x_{3,End}$$
$$Note:$$
$$K = \frac{K_0}{t}$$
$$Thus$$
$$t \to 0$$
$$n_3(x,t) \to u_0(x,t) = impulse$$

This is a diffusion solution. The diffusion goes from the initial boundary to the distant boundary where it stops. The initial boundary as shown above is the impulse like source with a volume of the initial number of cells at the initial site and the initial time.

The key factor here is the diffusion constant σ which will determine how the cells spread out from the initial source. We assume a HGPIN initiation with only proliferation and then a loss of localization with E cadherin loss and then a diffusion state.

There may also be some growth in this phase as well.

4. Region 4: This is the transition across the blood stream.

5. Region 5: Now we have a metastatic growth and diffusion in an environment facilitating and supporting it. The result is as follows:

$$n_5(x,t) = K_5 \exp(\lambda_5 t) \exp(-\frac{1}{2\sigma_5 t} x^2)$$

$$t > 0$$

$$x_{5,0} < x < x_{5,End}$$

This solution can be used in the following application to the analysis of biopsies. The above solution must be modified slightly for boundary conditions but generally excellent second order changes suffice.

4 A SIMPLE APPLICATION

Using the above example we can then ask if it can be applied directly to some simple cases. We examine the case of HGPIN as an example. Let us begin by presenting some basic data on the prostate. The following table is an example of the sampling and the percent of the cells sampled:

Diameter (um)[3]	30
Volume (um3)	14137.2
Volume (cc)[4]	1.41372E-08
Volume Prostate (cc)	40
Radius Prostate (cm)	0.82
Cells in Prostate	2,829,414,594
18 Gauge Biopsy Needle Diameter (mm)[5]	0.84
Area (mm^2)	0.55417824
Area (cm2)	0.005541782
Length (cm)	1.63
Volume Single Core (cc)	0.01
Cells Single Core	640,098
Number Cores	24
Number Cells	15,362,351
Percent Sampled	0.543%
Cells Wide	28
Cells Deep	544

Note that in a typical biopsy using 24 cores we sample only 0.5% of the prostate. Now the question is; given that sample, can one ascertain the presence of PCa and if so how early? If diffusion of cells has already begun, then how does that diffusion expand the cell detectability? How do we correlate Gleason score for example with diffusion?

We know that in this phase, typically a Gleason 5-6 stage, we have proliferation and diffusion creating what may appear as new but distorted glandular clusters. The cells are no longer well structures and the new "glands" are smaller and not well structured. This is the diffusive effect.

[3] See Kang et al.
[4] Size of normal prostate.
[5] Based upon standard clinical data. http://en.wikipedia.org/wiki/Needle_gauge_comparison_chart

$$n_3(x,t) = K_3 \exp(\lambda_{33} t) \exp(-\frac{1}{2\sigma t} x^2)$$

$$t > 0$$

$$x_{3,0} < x < x_{3,End}$$

$$Note:$$

$$K = \frac{K_0}{t}$$

$$Thus$$

$$t \to 0$$

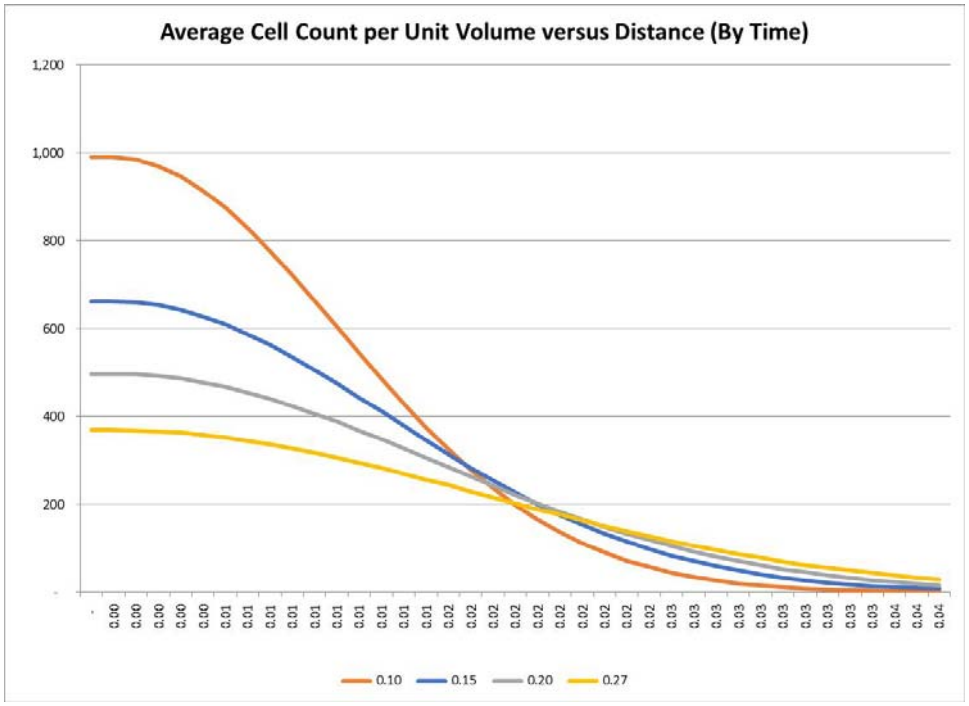
$$n_3(x,t) \to u_0(x,t) = impulse$$

Again we note that this is an aggregate average density per unit volume across the bounded space. Also this is an approximate solution since diffusion equations with dual boundaries can be solved precisely but the complexity is not worth the effort. Let us now consider a simple example.

We consider two cases.

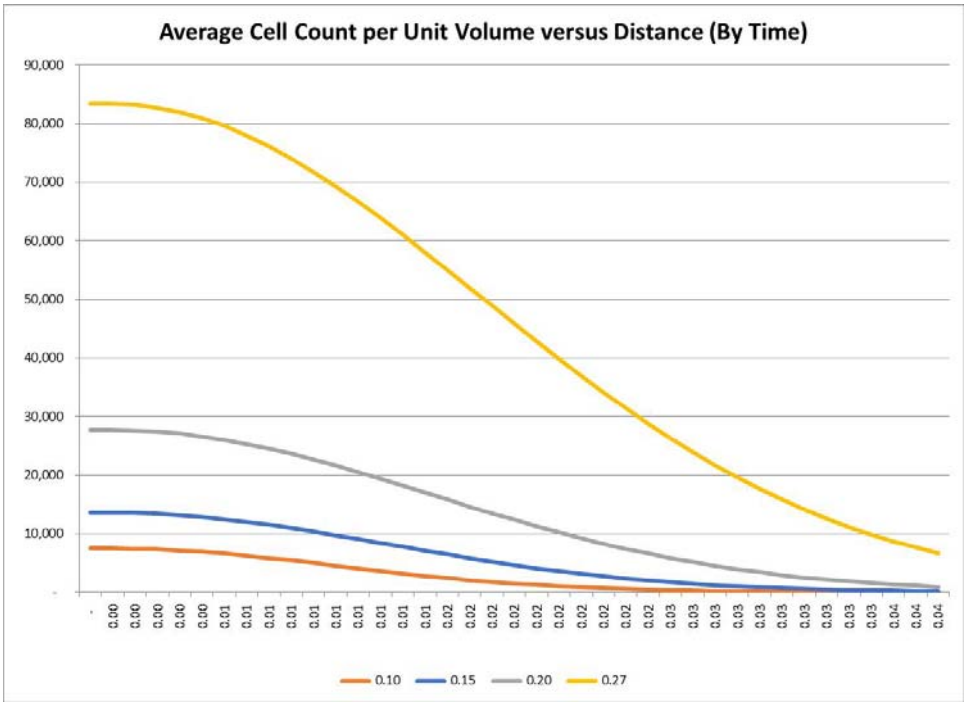
4.1 CASE 2: DIFFUSION ONLY

The case below assumes only diffusion. Note that we start with a localized cell count and then it becomes uniform across the band. But the count decreases.



4.2 CASE 1: GROWTH AND DIFFUSION

The Figure below demonstrates a typical growth and diffusion scenario. It shows that as time goes by the total cell density increases to an almost normalized level.



Thus we ask; what does this tell us about a biopsy sample? It tells a general story, which can be made specific as we obtain and use the parameters from data.

1. If we have diffusion only, then the spread is evened out over the range and decreases from the peak density at the start. The time required depends on certain diffusion constants. Here we look for aggressive and less aggressive mutations.

2. If the cells also grow and diffuse, we see not only diffusion but an increasing base as well. The growth will depend on the type of mutation of suppression.

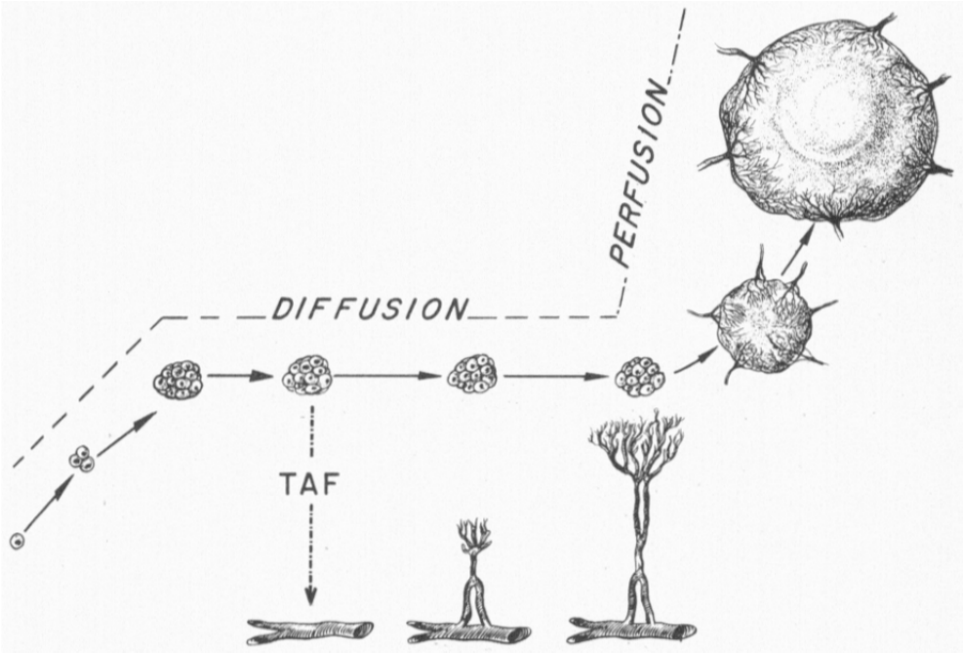
3. The question which we initially proposed was to determine what the detection and false alarm probabilities were for a biopsy. Namely if we have detected say HGPIN across the prostate on an initial biopsy, then on a repeat biopsy if we fail to detect any HGPIN what can we say about the state of the prostate? Have we just missed what was apparently quite pervasive? This can be quite questionable since HGPIN has been alleged to be prodromal of PCa. Furthermore the analysis we have performed would clearly indicate an expansion and not a contraction.

4. What are the profiles of PCa with various gene profiles. In the Gundem paper we have multiple profiles and there are also multiple clones. Our model allows for such a result and in fact strongly supports it.

5 INTRA AND EXTRAVASATION

The process of a malignant cell entering and exiting the blood stream is the process of intra and extravasation. These are complex processes and in the case of entry the cancer cell itself may create or promote the vascularization of itself, thus allowing entry and the exiting into selected organs can be complex. We briefly examine this process as one of the several that we have modelled above. There is an excellent summary of these issues in the work by Thiagalingam (see Chapter 18 by Ozturk et al). Also see Kerbel as well.

From Folkman's classic paper we have the following Figure:



Note that he used the term diffusion as we have adopted for the process of movement in the localized tumor. Then he uses perfusion, which we see as a modified flow mechanism. We will go through some examples of these processes later.

As Weidner has noted:

For a tumor to grow, the tumor cells must not only proliferate, but benign host tissue, especially new blood vessels, must also form around the tumor cells. In 1971, Folkman proposed that tumor growth is dependent on angiogenesis. Moreover, he has advocated that tumor cells and blood vessels compose a highly integrated ecosystem, that endothelial cells can be switched from a resting state to one of rapid growth by a diffusible signals from tumor cells, (or inflammatory cells or stroma-derived products), and that anti-angiogenesis might be an effective anticancer therapy. Indeed, there is abundant evidence that tumor growth and spread is angiogenesis dependent, that tumor cells can produce diffusible angiogenic regulatory molecules, and that

angiogenesis antagonists can slow or prevent tumor growth. Much of this evidence as well as probable tumor angiogenic mechanisms have been summarized in a various reviews

Angiogenesis is a fundamental process for malignant cells. The details of that process are somewhat known and involve modifications of cells to adapt to the new environment as well as to initiate and progress through the endothelial membranes of the vasculature. Also the tumor cells have the ability to recruit other cells to assist in this process.

5.1 INTRAVASATION

Intravasation is the progression of cells from a diffusive state to their entry into the blood stream. It also is the process of angiogenesis, the classic process observed by Folkman.

From Reymond et al we have recent summary of this process:

To intravasate, invading cancer cells first need to move to blood vessels, which can be within the tumour (neovasculature) or close to the tumour. Invasion through the stroma to blood vessels can be promoted by tumour-associated macrophages. Cancer cells can then enter the circulation by transmigrating either paracellularly through the endothelial cell (EC) junctions or transcellularly through the EC body. Matrix metalloproteinase 1 (MMP1) seems to be required for paracellular intravasation in regions where protease-activated receptor 1 (PAR1) on ECs mediates the remodelling of endothelial junctions. Alternatively, a disintegrin and metalloproteinase 12 (ADAM12) on ECs can induce cleavage of vascular endothelial cadherin (VE-cadherin) and angiopoietin 1 receptor (TIE2), which leads to disruption of endothelial junctions. Moreover, macrophages can attract cancer cells towards blood vessels by secreting epidermal growth factor (EGF) and can also secrete tumour necrosis factor 1α (TNF1α), which induces the retraction of endothelial junctions, thus facilitating cancer cell transendothelial migration (TEM).

Cancer cells can use Notch receptors to bind to Notch ligands on ECs and thereby transmigrate through the endothelial junctions; they can also secrete transforming growth factor β1 (TGFβ1), which induces the retraction of endothelial junctions. During transcellular intravasation, the Ca2+ –calmodulin complex in an EC activates myosin light chain (MLC) kinase (MLCK) at the sites of cancer cell attachment, which leads to local phosphorylation (P) of MLC and to actomyosin contraction. In turn, this leads to rapid cytoskeletal and membrane remodelling, which creates a transitory pore-like structure for the cancer cell to cross the EC.

As stated in Thiagalingam (pp283-285) the process of metastasis starts with intravasation. Cells such as tumor-associated macrophages are recruited to the site which in turn establishes a basis for neo-vascularization.

Weidner provides a discussion of an alternative view of this process. As the author states:

To metastasize, a tumor cell must gain access to the vasculature from the primary tumor, survive the circulation, escape immune surveillance, localize in the vasculature of the target organ,

escape from (or grow from within) the vasculature into the target organ, and induce tumor angiogenesis. Tumor spread is amplified when the new metastasis sheds additional tumor cells to form even more metastases by following the same sequence of events. Angiogenesis is needed; because, without it, tumor cells would not shed into the circulation. Greater numbers of tumor vessels increase the opportunity for tumor cells to enter the circulation. Using a transplantable mouse fibrosarcoma model….. showed that tumor cells shed into the bloodstream increased from roughly 103 cells per 24 hours on day 5 after tumor implantation to roughly 105 cells per 24 hours on day 15, an increase correlating closely with increasing intratumor microvessel density, especially when the intratumor microvessels were more than 30 microns in diameter (ie, sinusoid-sized blood vessels).

Also, these studies revealed that the establishment of lung metastases is directly related to the number of tumor cells (especially cell clusters) shed into the circulation. These elegant experiments were among the first to show that intratumor microvessel density can correlate with aggressive tumor behavior. Subsequently, many studies have shown that intratumoral microvessel density correlates with tumor aggressiveness of many different tumor types.

There is thus a complexity in intravasation which will need further clarification.

5.2 EXTRAVASATION

Extravasation is the process whereby the malignant cell leaves the blood stream and finds a location for growth. In PCa this is oftentimes in the bone. The question is; what is this process and how can it be modelled?

From Stoletov et al:

For a tumor cell to disseminate in the body it must perform several important steps, including invasion of surrounding tissues, intravasation into the blood vessel, survival in the circulation, extravasation from the blood stream, and proliferation at a secondary site in a foreign environment….Several studies have attempted to visualize cellular mechanisms of tumor cell extravasation in vivo using mouse or chick CAM human tumor xenograft assays.

These studies led to the conclusion that tumor cell extravasation is a complex process that might involve passive or active tumor cell movement within the vessel lumen, adhesion of tumor cells to the vascular wall and transendothelial passage of tumor cells using yet unknown mechanisms….In summary, our findings provide evidence that extravasation of tumor cells is influenced by specific metastatic gene signatures that alter the actin-myosin cytoskeleton and induce vasculature remodeling.

These findings challenge the widespread belief that tumor cell extravasation is a simple passive process and not a crucial determinant in the metastatic cascade. In light of these findings, a detailed understanding and confirmation of the regulatory mechanisms that govern tumor cell extravasation in zebrafish and mammals could provide valuable clinical markers that predict

metastatic potential and provide novel therapeutic targets designed to block the spread of cancer in patients.

The above is an indication that the process is complex and not yet well understood. The question for modelling is; can we examine some gross feature for extravasation as a gene change or some flow measure? We have assumed that such was the case; however, unlike most of the other metrics we do not have a clear path to gross measurement in this area. One can examine models for extravasation say as that of the neutrophil. Namely the cell just flows along until it is captured by some ligand in the vasculature which matches a receptor on the cell. Then the process of entering the local somatic region would occur. This is, however, speculation.

As Chen et al state:

Cancer cells disseminate in the body by undergoing several steps, including the invasion of surrounding tissues, intravasation into the blood vessel, transport in the circulation, extravasation from the vasculature and proliferation at a secondary site. Extravasation involves a cascade of events consisting of

(1) tumor cell arrest on the endothelium resulting in the formation of dynamic contacts that give rise to significant cytoskeletal changes, and

(2) tumor cell transendothelial migration (TEM) and subsequent invasion into the surrounding matrix.

Although the mechanisms of intravasation have been widely studied, the precise cellular interactions and molecular alterations associated with extravasation are poorly understood. In fact, most data are gathered from low-resolution in vivo studies and endpoint assays that indirectly observe tumor cells via quantification of secondary tumor formation in existing animal models. As such, direct observation of tumor cell arrest on and subsequent migration across an endothelium in a precisely controlled and physiologically relevant microenvironment would provide important insight into extravasation mechanisms

5.3 MODEL IMPACTS

The above discussion, limited as it may be, may lend more confusion than clarity. Our model for the complete process in intravasation-movement-extravasation is a simple flow mechanism. On the other hand the details from studies see a complex process of genetic expression change, cell recruitment, endothelial entry and exit, not to mention flow. Yet from the perspective of a gross model, this results in a simple form of flow, namely a time delay getting from point A to point B. The more compelling question is at this stage extravasation and selectivity. Namely; what is the detailed process whereby the cancer cell recognizes an organ specific location? How, for example, doe a prostate malignancy detect bone? Although a compelling question, we still argue that the "mechanics" of the cell getting from one location to another is still "flow".

As stated in DeVita et al:

After intravasation into the circulation from the primary tumor, tumor cells encounter significant physical stress from shear forces or mechanical arrest in small-diameter vessels. The hepatic sinusoids can be activated by the mechanical restriction of tumor cells to secrete nitric oxide. Nitric oxide can cause apoptosis of arrested tumor cells and has been shown to be required for the massive cell death of experimentally injected melanoma cells. Endothelial cells can also guard against wandering tumor cells through expression of DARC, a Duffy blood group glycoprotein.

DARC interacts with KAI1 expressed on circulating tumor cells causing them to undergo senescence. KAI1 was originally identified as a metastasis suppressor gene. The immune system can also actively attack circulating tumor cells.

For example, NK cells can engage cancer cells via TNF-related molecules such as TRAIL or CD95L, or through the perforin pathway. Both systems cause tumor cell death, and inhibiting TRAIL or using mice that are deficient in NK cells leads to increased metastasis. Because of these mechanical and cell-mediated stresses, the half-life of circulating tumor cells (CTCs) can be short. Estimations derived from the enumeration of CTCs before and after removal of the primary tumor in patients with localized breast cancer demonstrate that the half-life can be as short as a few hours.

How can CTCs evade cell death to enhance their metastatic potential? Growth at the primary tumor site will involve a selection for increased resistance to apoptosis due to cell death signals that are normally activated by inappropriate oncogene activation, tumor suppressor loss, or loss of cell-cell contact.

Antiapoptosis genes such as BCL2 or BCL-XL, or the loss of proapoptotic genes and downstream effector molecules belonging to the TNF-related receptor family such as CASPASE-8 can result in increased metastasis. Part of this may be because of survival both in the circulation and shortly after extravasation. Both CTCs and platelets can also express the αvβ3 integrin to promote aggregation of these cells to form tumor emoboli.

This aggregation not only facilitates arrest but can protect against shear forces and NK cell-mediated killing. Activation of αvβ3 has been shown to be required for formation of tumor emboli and metastasis in a breast cancer model.

The human body has so many defenses against the propagation of malignant cells, yet somehow this “flow” occurs. The flow is not commensurate with the flow rate of the blood stream but the ersatz flow through all of these barriers. Thus in determining the flow rate one must determine the effective transit time and potential loss therein of cells migrating from the initial point of entry to that of exit, We do not present a complete solution here but suggest a process.

6 SOME MIRNA FACTORS

The expression of genes can be influenced by a variety of factors. We examine two of recent study for the purpose of highlighting some of the complexities. Specifically we examine miRNA issues.

6.1 ALDH1A3

ALDH, aldehyde dehydrogenase, is a protein used to convert aldehydes into retinoic acid. Its pathway controls are not clearly delineated yet it has seen significance in PCa. We examine it again as a target for miRNA modification. It is a complex gene in terms of what specific functions its supports.

As LeMagnen et al note:

Notably, ALDH has been shown to be involved in stem cell protection and differentiation, and high levels of ALDH activity have been found in several stem cell populations. Thus, high ALDH activity has been used to select and identify normal hematopoietic stem cells and tumor-initiating cells (TIC) in hematopoietic malignancies.

From NCBI we have the following description of ALDH[6]:

This gene encodes an aldehyde dehydrogenase enzyme that uses retinal as a substrate. Mutations in this gene have been associated with microphthalmia, isolated 8, and expression changes have also been detected in tumor cells. Alternative splicing results in multiple transcript variants.

From Allahverdiyev et al:

ALDH1A3 is a cytosolic homodimer that participates in RA synthesis, oxidizes both alltrans-retinal and 9-cis-retinal (Km 0.2 μM for all-trans-retinal) to RA, and has an important role in embryonic development; including brain, retina, skeletal muscle, tooth buds, intestine, kidney, prostate, lung, liver and pancreas, it is expressed in various late-stage embryonic and adult rodent tissues. In humans, ALDH1A3 expression has been noted in stomach, salivary gland, breast, kidney and fetal nasal mucosa. Aldh1a3−/− mouse embryos die as a result of defects in nasal development.

It's been shown that ALDH1A3 takes part in the development of the eye, nucleus accumbens and olfactory bulbs, the forebrain, hair follicles and the cerebral cortex. ALDH1A3 deficiency has been shown to play a critical role in cancer by a number of studies. For instance, in human breast cancer MCF-7 cells, ALDH1A3 expression is downregulated, whereas in cultured human colon cancer cells, ALDH1A3 is one of two genes that are upregulated by induction of wild type p53.

[6] http://www.ncbi.nlm.nih.gov/gene/220

In mammary tumor-susceptible BALB/cJ mice that are heterozygous for p53, Aldh1a3 is one of five candidate genes located within a region determined for its linkage to mammary tumorigenesis. In mice resistant to induced mammary tumors, (C57BL/6J), Aldh1a3 is one of the two upregulated genes. ALDH1A3 is silenced by methylation in gastric cancer cells, whereas in glioblastoma cells, it is triggered by the antitumor agent IL-13 cytotoxin

As Floor et al state:

CSC–TPC are defined by their immortality, their capacity to reproduce all derived cell phenotypes of a cancer and by biological and biochemical markers (such as CD44, CD133, aldehyde dehydrogenase (ALDH), a high CD44/ CD24 ratio and so on). … High ALDH activity was shown to characterize hematopoietic stem cells and to select for TICs in various types of tumors, supporting a link between ALDH expression and carcinogenesis.

High ALDH activity was also proposed to select for highly tumorigenic cells in prostate cancer cell lines. To date, however, there is no evidence for the existence of ALDH… populations in primary prostate cancer. In the present work, we therefore investigated presence, prevalence, characteristics, and clinical relevance of ALDH… populations in primary prostate cancer.

Thus ALDH is associated with the critical stages of malignancy.

The blockage of ALDH by miRNAs is an example of pathway control mechanisms of miRNAs. A recent paper by Casanova-Salas et al has examined the impact of miR-187 and the ALDH1A3 expression. As they state:

This study used a proteomic approach based on two-dimensional gel electrophoresis (2D-DIGE) followed by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis and, for the first time, identified ALDH1A3 as a miR- 187 target in PCa. In addition, the potential utility of ALDH1A3 as a tumor biomarker was evaluated.
They continue:

In this study, 9 putative targets of miR-187 were identified by 2D-DIGE and MS analysis. From these we selected aldehyde dehydrogenase 1A3 (ALDH1A3) for further evaluation because it has been described to be regulated by androgens. Western blot analysis, qRT-PCR and IHC confirmed the direct regulation of ALDH1A3 by miR-187.

First, the inverse correlation between ALDH1A3 and miR-187 was confirmed by recovering miR-187 expression in PC-3, LNCaP and DU-145 cells, which led to a down-regulation of ALDH1A3 protein levels.

*Second, the inhibitory effect of miR-187 on ALDH1A3 expression was further confirmed by a luciferase reporter assay that showed a decrease in ALDH1A3 expression (*20% reduction in luciferase signal) upon miR-187 mimic transfection.*

Third, the inhibition of ALDH1A3 was observed when analysing a cohort of PCa human patient samples, both fresh and FFPE tissues. In these cohorts, the strong down-regulation of miR-187 was accompanied by an increased ALDH1A3 mRNA expression.

Fo(u)rth, the role of ALDH1A3 as miR-187 target was confirmed by IHC analysis.

Hence, ALDH1A3 was found to be up-regulated in prostate tumors and the expression of this protein is inversely correlated with the expression of the miRNA. In addition, the potential role of ALDH1A3 as candidate prognostic biomarker for PCa was evaluated, although in the cohort of samples analysed it did not provide any additional information. Nevertheless, the association of ALDH1A3 expression with Gleason score provides evidence of an increase in ALDH1A3 expression with tumor staging. We have previously postulated that loss of miR-187 during PCa progression could indicate a role as tumor suppressor.

Additionally, ALDH1A3 was found to cooperate with PSA in the prediction of the biopsy result. Apart from its association with the presence of the tumor in IHC of FFPE slides, we were able to measure ALDH1A3 in urine samples, finding a positive association with tumor appearance.

In this context, the identification of ALDH1A3 as a miR-187 target and its up-regulation in PCa indicates its potential role as an oncogene with an implication in PCa development.

Casanova-Salas et al conclude:

ALDH1A3 is a member of the human aldehyde dehydrogenase family that includes different subtypes ALDH1A1, ALDH1A2, ALDH1A6, etc. that catalyze the oxidation of retinal to retinoic acid (RA), which is required for normal prostate development. The implication of these enzymes in RA synthesis causes them to function as key enzymes in pathways associated with cell proliferation, differentiation and survival.

ALDH1A3 has been found to play a role as a predictor of metastasis in breast cancer. ALDH1A isozymes, mainly ALDH1A1 and ALDH1A3, have been also described as markers of cancer stem cells in different tumors and key determinants for the survival and drug resistance of cancer cells. In agreement with the association with stemness of ALDH1A3, miR-187 has been recently identified as a miRNA that specifically characterizes human embryonic stem cells and induces pluripotent stem cells.

Therefore both genes, miRNA and target, seem to regulate pluripotent cell characteristics which are related with a more undifferentiated and aggressive tumor phenotype. In this regard, recent results show that high ALDH activity can be also used to isolate human prostate cancer cells with significantly enhanced tumorigenicity and metastatic behavior. Thus, using a FACS sorting kit such as ALDEFLUOR, which classifies cells according to ALDH activity, might be a useful tool for the stratification of prostate cancer patients at risk of developing metastatic disease

Thus we know that ALDH1A3 is also an activator of stem cells. The issue here is that we have not incorporated expressly any stem cell characteristics in our models.

From Trasino et al[7]:

Previous gene array data from our laboratory identified the retinoic acid (RA) biosynthesis enzyme aldehyde dehydrogenase 1A3 (ALDH1A3) as a putative androgen-responsive gene in human prostate cancer epithelial (LNCaP) cells. In the present study, we attempted to identify if any of the three ALDH1A/RA synthesis enzymes are androgen responsive and how this may affect retinoid-mediated effects in LNCaP cells. We demonstrated that exposure of LNCaP cells to the androgen dihydrotestosterone (DHT) results in a 4-fold increase in ALDH1A3 mRNA levels compared with the untreated control.

The mRNA for two other ALDH1A family members, ALDH1A1 and ALDH1A2, were not detected and not induced by DHT in LNCaP cells. Inhibition of androgen receptor (AR) with both the antiandrogen bicalutamide and small interfering RNA for AR support that ALDH1A3 regulation by DHT is mediated by AR.

Furthermore, specific inhibition of the extracellular signal–regulated kinase and Src family of kinases with PD98059 and PP1 supports that AR's regulation of ALDH1A3 occurs by the typical AR nuclear-translocation cascade. Consistent with an increase in ALDH1A3 mRNA, DHT-treated LNCaP cells showed an 8-fold increase in retinaldehyde-dependent NAD+ reduction compared with control.

Lastly, treatment of LNCaP with all-trans retinal (RAL) in the presence of DHT resulted in significant up-regulation of the RA-inducible, RA-metabolizing enzyme CYP26A1 mRNA compared with RAL treatment alone.

Taken together, these data suggest that

(i) the RA biosynthesis enzyme ALDH1A3 is androgen responsive and

(ii) DHT up-regulation of ALDH1A3 can increase the oxidation of retinal to RA and indirectly affect RA bioactivity and metabolism

From LeMagnen et al,

Aldehyde dehydrogenase (ALDH) enzyme is responsible for the oxidization of cellular aldehydes resulting in the production of retinoic acid (3). Notably, ALDH has been shown to be involved in stem cell protection and differentiation, and high levels of ALDH activity have been found in several stem cell populations (4, 5). Thus, high ALDH activity has been used to select and identify normal hematopoietic stem cells (4, 6) and tumor-initiating cells (TIC) in hematopoietic malignancies (7). TICs, functionally defined as cells capable of initiating tumors in immunodeficient mice (8), have also been identified in a variety of human solid tumors (8–11). In

[7] http://ebm.sagepub.com/content/232/6/762.abstract

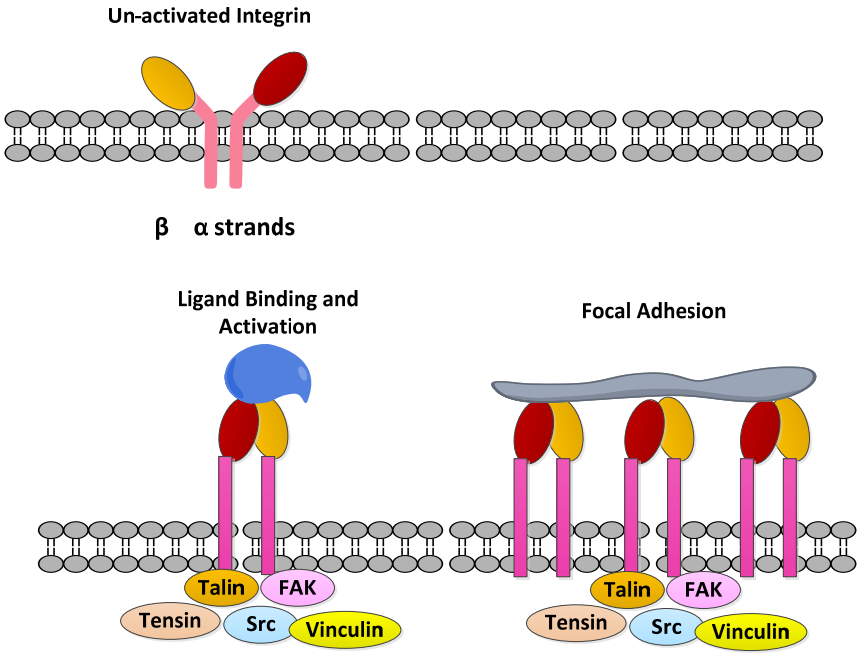
a number of cancers of diverse histologic origin, TICs have been reported to exhibit high levels of ALDH activity (5, 12–14).

6.2 TALIN

Talin is a protein which works with integrins in stabilizing a cell with the ECM. It appears that on the one hand talin facilitates binding while on the other hand talin can facilitate metastatic movement. We examine a specific protein, talin1, and more explicitly we examine some recent results of the impact of this protein by miRNAs. This specific example is an interesting conjunction of integrins, the ECM, the possible intravasation process. It also includes a loss of apoptosis as well. As such this specific example deals with proliferation, diffusion, and the commencement of a putative flow mechanism. More importantly this process demonstrates the effects of miRNAs rather than the change of a purely genetic nature.

As Kyprianou has noted (pp 85-87), an overexpression of talin1 leads to an activation of FAK/AKT signalling through both ECM dependent and independent means. The activation of AKT is directly linked to resistance to anoikis, the apoptosis due to loss of cell anchorage (Kyprianou p3) and thus a survival of the cell as it becomes detached and begins the process of movement in the prostate and eventually an intravasation.

Let us start by a quick review of integrins, talin, and the ECM interactions. We demonstrate this below. Integrins are a combination of two strands, an α and β strand. Integrins come in a multiplicity of types depending upon the α and β strand combinations. They span the intercellular and extracellular space and there may be often multiple types of integrins on a single cell. The integrins can be activated by first a locations of certain proteins in the cytoplasm and then when activated an act as receptors for ligands and thus activate pathways in a cell (see Zent and Pozzi, pp 29-36). One of the activating proteins is Talin which we will discuss shortly. Also once activated they also bind to proteins in the ECM as well. We demonstrate one type of ECM connection via a focal adhesion.



To understand this adequately we refer to the work by Desiniotis and Kyprianou who state:

Upon detachment from the extracellular matrix, tumor epithelial cells and tumor-associated endothelial cells are capable of overcoming anoikis, gain survival benefits, and hence contribute to the process of metastasis.

The focal-adhesion complex formation recruits the association of key adaptor proteins such as FAK (focal-adhesion kinase).

Vimentin, paxillin, and talin are responsible for mediating the interaction between the actin cytoskeleton and integrins.

Talin is an early-recruited focal-adhesion player that is of structural and functional significance in mediating interactions with integrin cytoplasmic tails leading to destabilization of the transmembrane complex and resulting in rearrangements in the extracellular integrin compartments that mediate integrin activation.

Talin-mediated integrin activation plays a definitive role in integrin-mediated signaling and induction of downstream survival pathways leading to protection from anoikis and consequently resulting in cancer progression to metastasis.

We recently reported that talin expression is significantly increased in prostate cancer compared with benign and normal prostate tissue and that this overexpression correlates with progression to metastatic disease implicating a prognostic value for talin during tumor progression.

At the molecular level, talin is functionally associated with enhanced survival and proliferation pathways and confers anoikis resistance and metastatic spread of primary tumor cells via activation of the Akt survival pathway. In this review, we discuss the growing evidence surrounding the value of talin as a prognostic marker of cancer progression to metastasis and as therapeutic target in advanced prostate cancer, as well as the current understanding of mechanisms regulating its signaling activity in cancer.

The specific recognition of the anoikis resistance is essential. Normally when a cell loses its adhesive capability it starts to wander and an apoptotic process of anoikis takes over the cell dies. However in a cancer cell this movement must be supported by an anoikis avoidance and talin seems to affect that. Let us briefly examine the integrins in some further detail. From Wang we have a brief summary:

By regulating cell extracellular matrix contact, cell-cell adhesion and cell-pathogen interaction, integrins take part in a wide-range of biological processes, including development, angiogenesis, immune response, cancer and hemostasis, etc.

The name of integrin inherently means to ***integrate the extracellular and intracellular environments***.

There comes the most important aspect of their function: the bidirectional signaling across the plasma membrane. On one hand, ligand binding to the integrin extracellular domains is transduced to the cytoplasm in the classical "outside-in" direction to regulate intracellular activities.

On the other hand, integrins are often expressed on the cell surface in a default low-affinity ligand-binding conformation. When cells become activated, for example by cytokine, integrins are rapidly activated in response to cellular stimulation within the cytoplasm. They undergo large conformational changes, resulting in a dramatic affinity increase for ligand binding

Now as to detailed functionality of integrins and their signalling effects we have from Guo et al:

Integrins signal predominantly through the recruitment and activation of Src-family kinases (SFKs). Most integrins recruit focal adhesion kinase (FAK) through their beta subunits. As well as activating signalling from phosphatidylinositol 3-kinase (PI3K) to AKT/protein kinase B (PKB) through phosphatidylinositol-3,4,5-trisphosphate, FAK functions as a phosphorylation-regulated scaffold to recruit Src to focal adhesions.

FAK is an integral player with talin. In fact we see FAK and the more significant of the two but talin is essential as an activator. They continue:

Here, Src phosphorylates p130CAS and paxillin, which recruits the Crk–DOCK180 complex, and thereby results in the activation of Rac. Rac then leads to the activation of p21-activated kinase (PAK), Jun amino-terminal kinase (JNK), and nuclear factor kappaB (NF-kappaB. FAK also activates extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase

(MAPK) by recruiting the RAP1 guanine nucleotide-exchange factor (GEF) C3G through Crk. RAP1 then activates ERK/MAPK through B-Raf.

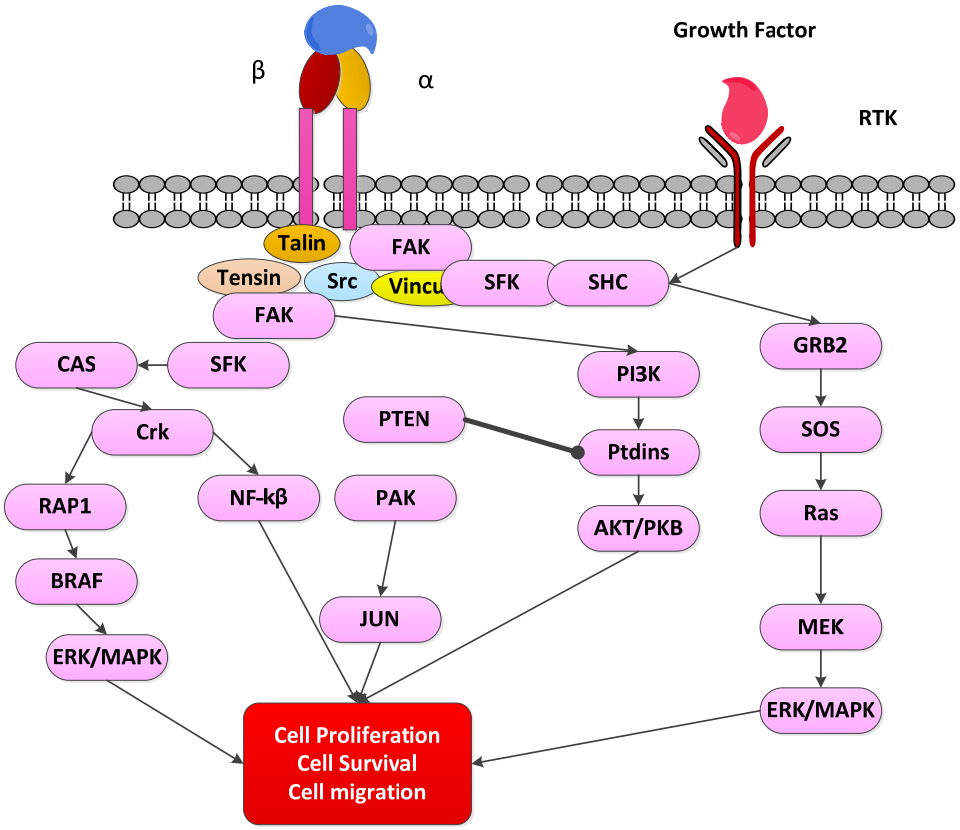
BRAF is a significant factor in many malignancies. We know its key role when mutated in melanoma via BRAF V600.

Alternatively, FAK can activate ERK/MAPK by recruiting the growth-factor-receptor-bound-2 (GRB2) and son-of-sevenless (SOS) complex. Certain integrins, including alpha5beta1, alpha1beta1 and alphavbeta3, are coupled to palmitoylated SFKs, such as Fyn and Yes, through their alpha subunits. In this pathway, caveolin-1 functions as a transmembrane adaptor to facilitate the recruitment of Fyn and Yes. The palmitoylated SFKs recruit and phosphorylate the adaptor SHC, which combines with GRB2–SOS to activate ERK/MAPK signalling from Ras91, 124. Some integrins can also directly interact with SFKs through the cytoplasmic domain of their beta subunits125.

One integrin, alpha6beta4, is palmitoylated, and it combines with SFKs that are similarly palmitoylated in LIPID RAFTS126. The SFKs phosphorylate several tyrosine residues in the cytoplasmic domain of beta4, which causes the recruitment of SHC and activation of Ras–ERK/MAPK and PI3K signalling. The pathways that integrins activate through SFKs are sufficient to induce cell migration and to confer some protection from apoptosis on cells.

Thus management of integrins for stability would be essential in maintaining normal cell growth and death.

We show from the previous reference the pathways (as modified) and the end results related thereto.



Now Stegeman et al have recently published a paper examining PCa and miRNAs. Our concern here is how miRNAs can affect changes in the integrin ECM control. As they note:

miRNAs negatively regulate their target mRNAs primarily through Watson–Crick base-pairing interactions. The most critical region for mRNA binding and repression is miRNA nucleotides 2 to 8, referred to as the miRNA seed site. Experiments have shown that genetic variations within the seed site or in the target mRNA at sites complementary to miRNA seed sites, referred to as miRSNPs, may reduce effectiveness or abolish miRNA-mediated repression, having functional consequences for cancer risk.

Now in a recent paper by Zhang et al we have an interesting mix of integrins, miRNAs, talin, and the beginnings of diffusive movement in PCa. Our focus is twofold. First, we desire to examine this study briefly to have an understanding of the process. Second, we want to see how this micro level process can be reflected into the gross model of our approach. This presents a complex

mechanism for what we have termed as diffusion. There is no genetic change in a controlling gene but a change in expression of a controlling miRNA.

As Zhang et al state:

MicroRNAs (miRNAs) are a class of small (19–25 nt) non-coding RNAs that play important roles in gene regulation by partial or full complementary matching with the 3'-untranslated region (UTR) of target mRNAs and triggering transcriptional or post-transcriptional suppression. They are involved in numerous physiological functions such as cell differentiation, migration, proliferation, apoptosis, and senescence. MiR-124, a putative tumor suppressor located in 8q12.3, is frequently found to be down-regulated in several human malignancies including bladder cancer, hepatocellular carcinoma, breast cancer, glioma, glioblastoma, cholangiocarcinoma, gastric cancer, osteosarcoma, ovarian cancer, and prostate cancer. The biological effects of miR-124 in tumor cells are mainly negative regulation of cell proliferation, apoptosis, and especially metastasis.

They conclude that miR124 suppresses talin1 and that excess talin1 over excites the pathways and results in loss of anoikis and thus a metastatic growth.:

Src-FAK signaling has been recently reported to promote E-cadherin internalization, which facilitates tumor cell motility, inhibits the endocytic pathway, and activates MMP2 and MMP9 during cancer progression. Accordingly, we identified suppression of effector proteins related to tumor invasion, MMP2, MMP9, and E-cadherin, in miR- 124-transfected tumor cells. These results might illustrate how miR-124 confers non-migratory and non-invasive phenotypes to prostate cancer cells by targeting talin 1 in an integrin-dependent manner…

Collectively, our study has shed light on the anti-migration and anti-invasion mechanisms of miR-124 in prostate cancer. Our preliminary experiments suggest that talin 1 is very likely a novel player in the anti-metastatic signaling network of miR-124, following ROCK, Rac-1, and transforming growth factor-α. Considering the limitations of in vitro experiments, further in vivo investigations are needed to confirm these results.

7 TGF AND PCA

We now examine another dimension of cancer development, that of growth factors. We focus on TGF, the Transforming Growth Factor because of some recent work reported regarding PCa. TGF-β1 is a cytokine that regulates development and also functions to preserve homeostasis in developed tissues[8]. As NCBI states[9]:

This gene encodes a member of the transforming growth factor beta (TGFB) family of cytokines, which are multifunctional peptides that regulate proliferation, differentiation, adhesion, migration, and other functions in many cell types. Many cells have TGFB receptors, and the protein positively and negatively regulates many other growth factors. The secreted protein is cleaved into a latency-associated peptide (LAP) and a mature TGFB1 peptide, and is found in either a latent form composed of a TGFB1 homodimer, a LAP homodimer, and a latent TGFB1-binding protein, or in an active form composed of a TGFB1 homodimer. The mature peptide may also form heterodimers with other TGFB family members.

TGF-β1 is a key gene in maintaining normal cell growth. Loss of its function is related to metastasis. We look at this gene and its related genes SMAD as one of the changes we would see in metastasis. Note here that we are suppressing a gene by the over expression of another. Previously we saw miRNAs as the control factor, here we see another gene.

As stated in Thiagalingam (pp283-284) activation of the TGFβ pathway results in the loss of E cadherin and other factors and activation of master regulators. Similar to the integrin reactions with talin this allows for migration of the tumor cells.

Specifically we examine the work by Fournier et al[10] which indicates that *TGF-β inhibition* decreases prometastatic genes and prostate cancer bone metastases. Moreover the authors indicate a mechanism whereby PMEPA1 inhibits TGF-β signaling by a non-proteasomal mechanism. This then implies that in a clinical setting a low level of PMEPA1 correlates with "poor metastasis-free survival". The conclusion is then that PMEPA1 knockdown increases prostate cancer bone metastases in the mouse model they examined. They specifically state:

Transforming growth factor-β (TGF-β) regulates the expression of genes supporting breast cancer cells in bone, but little is known about prostate cancer bone metastases and TGF-β. Our study reveals that the TGFBR1 inhibitor SD208 effectively reduces prostate cancer bone metastases. TGF-β upregulates in prostate cancer cells a set of genes associated with cancer aggressiveness and bone metastases, and the most upregulated gene was PMEPA1. In patients, PMEPA1 expression decreased in metastatic prostate cancer and low Pmepa1 correlated with decreased metastasis-free survival.

[8] See Bunz pp 198-201.

[9] http://www.ncbi.nlm.nih.gov/gene/7040

[10] http://www.cell.com/cancer-cell/abstract/S1535-6108%2815%2900142-7

Only membrane-anchored isoforms of PMEPA1 interacted with R-SMADs and ubiquitin ligases, blocking TGF-β signaling independently of the proteasome. Interrupting this negative feedback loop by PMEPA1 knockdown increased prometastatic gene expression and bone metastases in a mouse prostate cancer model.

For the gene PMEPA1, or the "prostate transmembrane protein, androgen induced 1", we have from NCBI[11]:

This gene encodes a transmembrane protein that contains a Smad interacting motif (SIM). Expression of this gene is induced by androgens and transforming growth factor beta, and the encoded protein suppresses the androgen receptor and transforming growth factor beta signaling pathways though interactions with Smad proteins. Overexpression of this gene may play a role in multiple types of cancer. Alternatively spliced transcript variants encoding multiple isoforms have been observed for this gene.

We examine SMAD later in this section but this is an example of a complex regulatory network which plays an integral role in PCa bone metastasis. To expand on the recent work we have from MedicalXpress[12]:

The researchers homed in on a protein that is essential in multiple cell functions such as cell growth and proliferation and, in some cases, natural cell death. The protein, TGF-beta, also has been found to promote bone metastasis in patients with breast cancer and melanoma.

By analyzing the genes present in patients with advanced disease, the researchers focused on the protein PMEPA1, which is abundant in primary prostate cancer cells but less common in advanced disease, including metastatic bone tumors.

To investigate the clinical significance of PMEPA1, the researchers compared its presence in normal tissue to primary tumors, finding that the gene was active in prostate, breast and lung cancer tumors. The opposite was true of TGF-beta, which led the researchers to determine that the presence of TGF-beta regulates the activity of PMEPA1.

"Comparing data on patients with prostate or breast cancer, we found those with low amounts of PMEPA1 developed metastases faster and had shorter survival," Dr. Guise said. "By inhibiting TGF-beta, we believe we could reduce the spread of prostate cancer to the bone and increase survival."

Drs. Guise and Fournier think that with additional analysis, the presence of PMEPA1 may serve in the future as a diagnostic tool to predict the likelihood of prostate cancer metastases and serve as an indicator of survival, similar to the Gleason score and PSA counts currently used by physicians to stage prostate cancer and determine options for treatment.

[11] http://www.ncbi.nlm.nih.gov/gene/56937

[12] http://medicalxpress.com/news/2015-05-focus-potential-tool-survival-staging.html

Namely, on the one hand a high PMEPA1 was in localized PCa cells and suppressed TGF whereas it was low in the metastatic cells and thus TGF was over-expressed, and this they considered a source of metastatic growth. To add to this we examine the work from Liu et al:

The PMEPA1 gene has been shown to suppress the androgen receptor (AR) and TGFβ signaling pathways and is abnormally expressed in prostate tumours. However, the role and mechanism action of PMEPA1 in AR-negative prostate cancer are unclear. Here, we demonstrate that inhibition of PMEPA1 suppresses AR-negative RWPE1 and PC-3 prostate cell proliferation through up-regulating the p21 transcription.

Additionally, PMEPA1 overexpression suppresses the p21 expression and promotes cell proliferation. PMEPA1 is induced by TGFβ as a negative feedback loop to suppress Smad3 phosphorylation and nuclear translocation; up-regulates c-Myc; down-regulates p21; and promotes PC-3 cell proliferation. The PMEPA1 functions depend on its Smad2/3 binding motif. Consistently, depletion of Smad3/4, but not Smad2, blocks PMEPA1's functions of regulating c-Myc and p21.

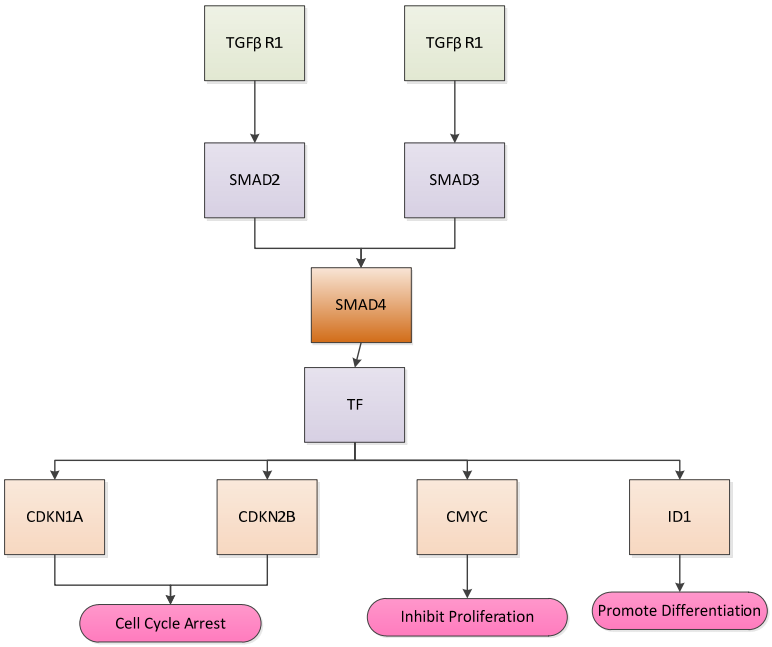
Importantly, stable depletion of PMEPA1 in PC-3 inhibits xenograft growth. Finally, we found that PMEPA1 is overexpressed in a subset of prostate cancer cell lines and tumours. These findings suggest that PMEPA1 may promote AR-negative prostate cancer cell proliferation through p21.

Thus the excess expression of PMEPA1 was at the heart of this process.

We now briefly consider SMAD so that we can have an understanding of the totality of this process. SMAD4 is an element in the TGF-β signalling chain we have just discussed. TGF is as indicated a cytokine, specifically a transforming growth factor cytokine. Like the Wnt-Apc pathway, the TGF pathway links defective development to cancer. The pathway is shown in part below (from Bunz p 199). Normal TGF signalling down-regulates the growth of most normal cells. Several of the genes in the TGF/SMAD pathway activation suppress growth. Specifically the genes CDKN1A and CDKN2B encode the cyclin dependent kinase inhibitors which suppress growth. Activated SMAD pathways also appear to suppress the transcription of other genes including c-Myc.

Gene	***Function***	***Disease***	***Pathway***
EWSR1	Translocation	Ewing's sarcomas, lymphomas, leukemias	SMAD
RUNX1	Translocation	Leukemias	SMAD
SMAD2	Inactivating codon change	Colon, breast	SMAD
TGFBR1, TGFBR2	Inactivating codon change	Colon, stomach, ovarian	SMAD

We show some of the TGF SMAD signalling below. We will elaborate this later.



SMAD4 controls the G1 to S transition. As stated in NCBI[13]:

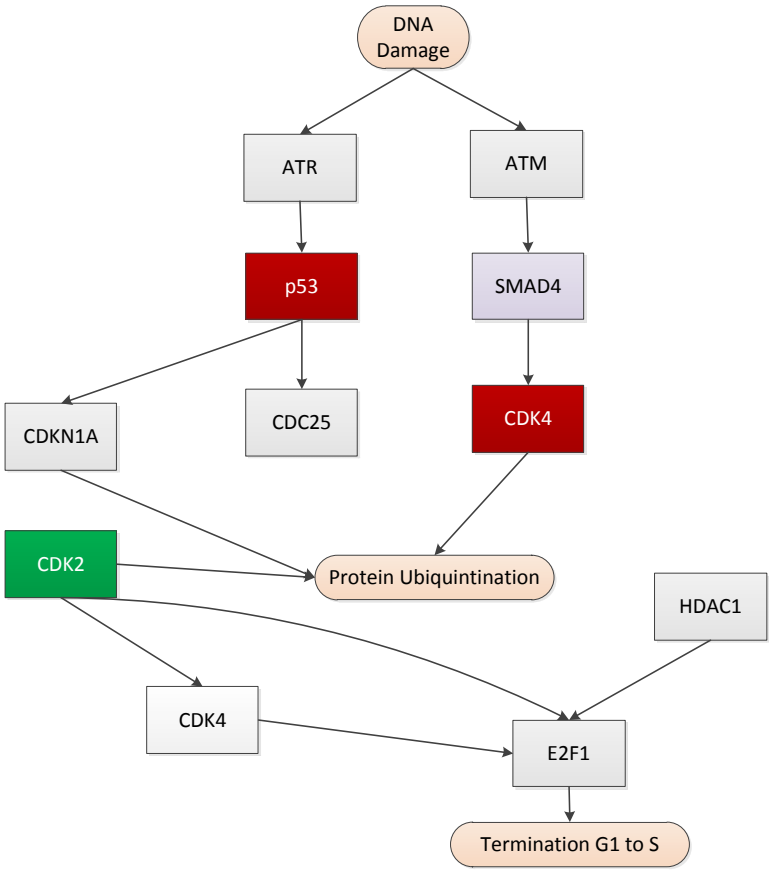
This gene encodes a member of the Smad family of signal transduction proteins. Smad proteins are phosphorylated and activated by transmembrane serine-threonine receptor kinases in response to TGF-beta signaling. The product of this gene forms homomeric complexes and heteromeric complexes with other activated Smad proteins, which then accumulate in the nucleus and regulate the transcription of target genes.

This protein binds to DNA and recognizes an 8-bp palindromic sequence (GTCTAGAC) called the Smad-binding element (SBE). The Smad proteins are subject to complex regulation by post-translational modifications. Mutations or deletions in this gene have been shown to result in pancreatic cancer, juvenile polyposis syndrome, and hereditary hemorrhagic telangiectasia syndrome.

We use the NCI data set for its pathway[14]:

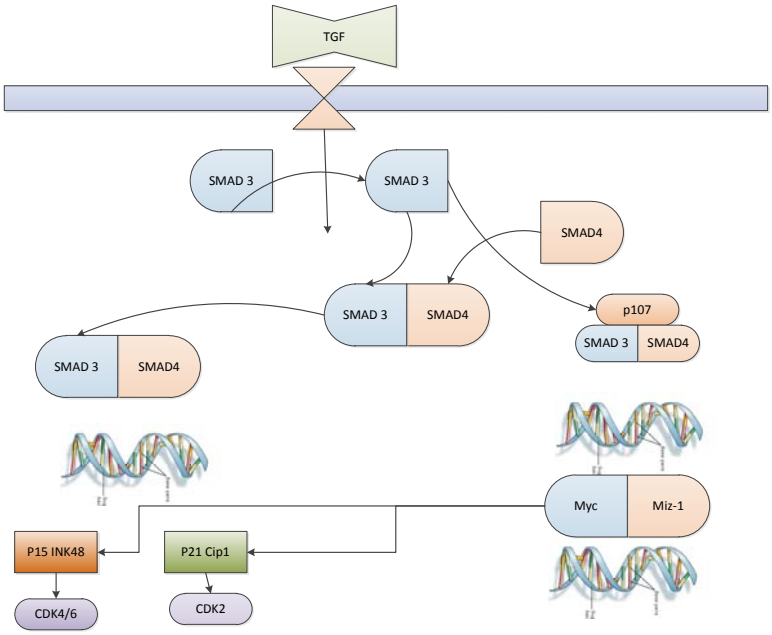
[13] http://www.ncbi.nlm.nih.gov/gene/4089

[14] http://pid.nci.nih.gov/search/pathway_landing.shtml?pathway_id=100160&source=BioCarta&genes_a=4089&genes_b=&what=graphic&jpg=on&ppage=1



The SMAD pathway is also detailed by NCI and one is referred to that source for further detail.

From Weinberg (p 291) we also have the SMAD4 pathway showing its immediate control of the DNA transcription.



As Weinberg states (p 292):

"… Half of all pancreatic carcinomas and more than a quarter of all colon carcinomas carry mutant inactivated Smad4 proteins. Without the presence of Smad4 neither Smad2-Smad4 nor Smad3-Smad4 complexes can form. These two complexes are the chief agents dispatched by the TGF-β receptor to the nucleus with the important assignment to shut down proliferation."

This control mechanism is shown above.

8 MULTIPLE GENE CHANGES

We often focus on single gene changes in expression. However there is a continual flow of reports detailing multiplicity of genes and their relevance. In modelling PCa one must try to establish operative changes and not pandemic ones. As we discussed in the past two sections, we may have miRNA changes or changes in genes which control the expression of others. Detailing the networks of these processes is complex if not in some cases futile. We examine another recent contribution, one called by the Press as a "Rosetta Stone".

In recent work by Robinson et al[15] they report that during a multi-institutional integrative clinical sequencing of mCRPC (namely metastatic castrate resistant PCa) that approximately 90% of mCRPC harbor clinically actionable molecular alterations. That is the alterations can be changed to their original state by some available or soon to be available therapeutic mechanism. Moreover they note that mCRPC harbors genomic alterations in several specific genes, namely PIK3CA/B, RSPO, RAF, APC, β-catenin, and ZBTB16. Finally they noted that 23% of mCRPC harbor DNA repair pathway aberrations, and 8% harbor germline findings. Robinson et al state:

Toward development of a precision medicine framework for metastatic, castration-resistant prostate cancer (mCRPC), we established a multi-institutional clinical sequencing infrastructure to conduct prospective whole-exome and transcriptome sequencing of bone or soft tissue tumor biopsies from a cohort of 150 mCRPC affected individuals.

Aberrations of

AR,
ETS genes,
TP53, and
PTEN

were frequent (40%–60% of cases), with TP53 and AR alterations enriched in mCRPC compared to primary prostate cancer.

Now the above genes are well known in PCa and have been extensively studied. There is no surprise here. They continue:

We identified new genomic alterations in

PIK3CA/B,
R-spondin,
BRAF/RAF1,
APC,
β-catenin, and
ZBTB16/PLZF.

[15] http://www.cell.com/cell/abstract/S0092-8674%2815%2900548-6

These genes also do not surprise us. PIK3CA is in the pathway controlling proliferation, APC is obvious as an AR related gene, and catenin is well known for cell motility control. They continue:

Moreover, aberrations of

BRCA2,
BRCA1, and
ATM

were observed at substantially higher frequencies (19.3% overall) compared to those in primary prostate cancers. 89% of affected individuals harbored a clinically actionable aberration, including 62.7% with aberrations in AR, 65% in other cancer-related genes, and 8% with actionable pathogenic germline alterations. This cohort study provides clinically actionable information that could impact treatment decisions for these affected individuals.

The BRCA changes were specifically enlightening. In a Science Daily report on the Robinson work they state[16]:

Nearly two thirds of the men in the study had mutations in a molecule that interacts with the male hormone androgen which is targeted by current standard treatments -- potentially opening up new avenues for hormone therapy.

Mutations in the BRCA1 and BRCA2 genes -- most famous for their roles in breast cancer -- were found in nearly 20 per cent of patients. Recent work at The Institute of Cancer Research (ICR) and The Royal Marsden has shown that these patients can be treated effectively by drugs called PARP inhibitors.

Researchers also discovered new mutations, never detected before in prostate cancer, but which do occur in other cancers. These include mutations in the PI3K and RAF gene families which can also be targeted by existing drugs, either currently in trials or approved for use in the clinic.

The researchers also took blood tests to analyse patients' own genomes, and found that 8 per cent were born with DNA errors that predisposed them to prostate cancer.

They said this could strengthen the case for genetic screening for people with a family history of the disease.

Previous genetic studies on prostate cancers had mostly analysed tissue from the primary tumours, which tend to carry fewer mutations than metastatic sites.

[16] http://www.sciencedaily.com/releases/2015/05/150521133732.htm

Studies of metastatic sites had been small and mostly used tissue taken during post mortems -- whereas in this study doctors took needle biopsies taken from patients during the course of their treatment.

Thus this work appears to re-establish for many of the genes noted further evidence of their importance. BRCA1,2 were noted as some others have done in the past. For example in Irshad et al 2013 we have a well-worn list of target genes. However the "Rosetta Stone" appellation may very well be a road too far. We clearly have detailed knowledge of the genes indicated, even some preliminary BRCA expression concerns. The key question is; now that we know this what can we do[17]?

[17] For example one can look at the work of Irshad, Gunmen and others and see the ever increasing proliferation of target genes to control. The problem is that there are so many that control may be futile. We see in metastatic melanoma that even with BRAF control that MEK pathways are activated and other cancers arise.

9 ESTIMATING CONSTANTS

How do we estimate the constants that we presented? A recent study by Stein et al made some extensive attempts ate estimating these rates. However one would need considerably more detail by genetic profile type. There generally is little if any data on such growth rates.

From Milo and Phillips we have:

Cell type	***Turnover time***
Small intestine epithelium	2-4 days
Stomach	2-9 days
Blood Neutrophils	1-5 days
White blood cells Eosinophils	2-5 days
Cervix	6 days
Crypts in colon	7 days
Lungs alveoli	8 days
Platelets	10 days
Bone osteoclasts	2 weeks
Intestine Paneth cells	20 days
Skin epidermis cells	10-30 days
Pancreas beta cells (mouse)	20-50 days
Blood B cells (mouse)	4-7 weeks
Trachea	1-2 months
Hematopoietic stem cells	2 months
Sperm (male gametes)	2 months
Bone osteoblasts	3 months
Red blood cells	4 months
Liver hepatocyte cells	0.5-1 year
Fat cells	8 years
Cardiomyocytes	0.5-30% per year
Skeleton	10% per year
Central nervous system	Life time
Lens cells	Life time

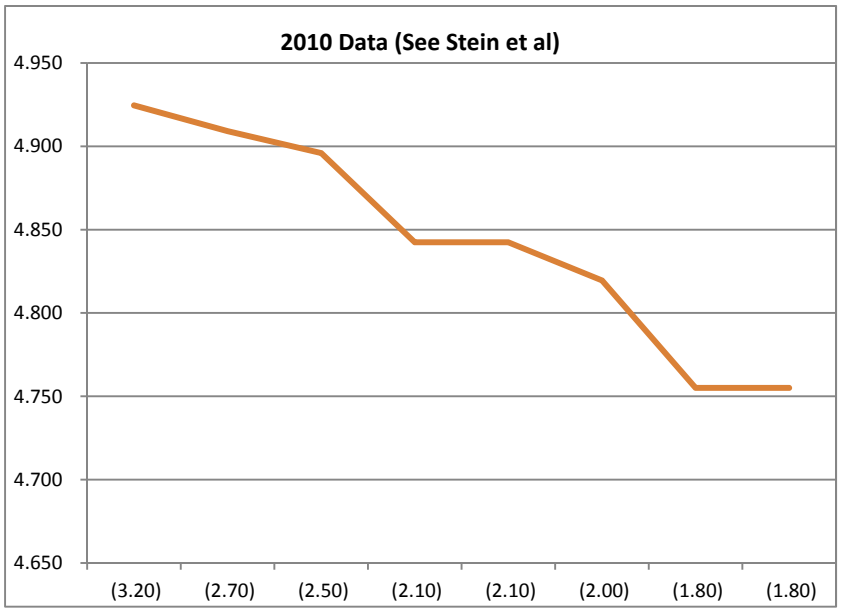
Now there have been multiple studies of growth rates of malignant prostate cells, see Stein as an example. We take that data and we attempt to relate it to what we have proposed.

Below is a summary of the NCI Stein data from 2010 and 2012. Simply, they determine the doubling time in days, noted as t below. Note that this is done for a set of protocols, some pretreatment and some post treatment. The authors also use PSA as a surrogate measure. Furthermore we have no knowledge as to the genetic makeup of the neither tumor nor do we have any knowledge of the location of the tumor.

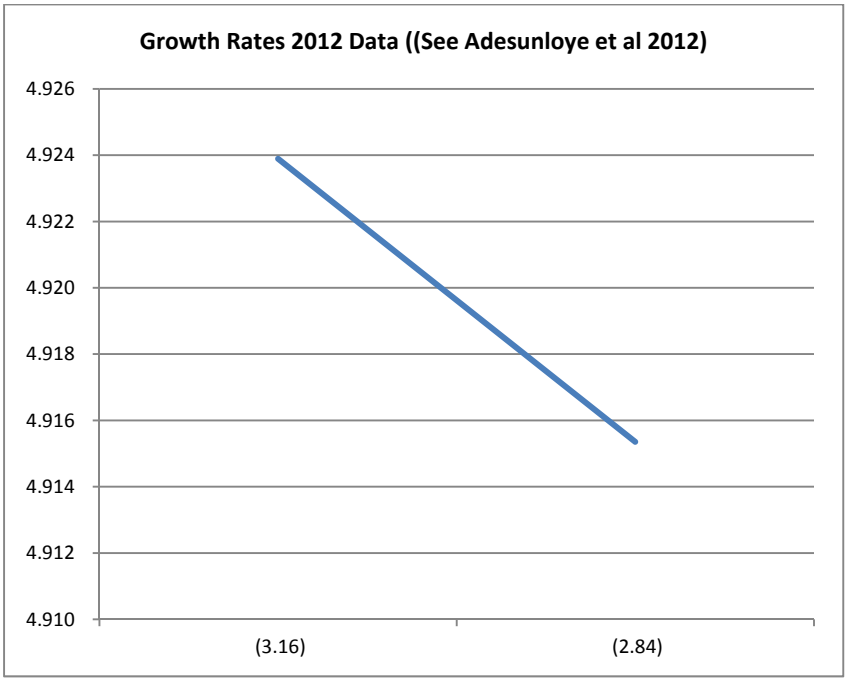
Protocol	Log (g)	T1 t (days)	T2 t (days)	Log (t)	Doubling Time t days
Stein et al 2010					
ATTP Post	(3.20)	0.693	0.001	0.692	4.925
Ketoconazole Alendronate	(2.70)	0.693	0.002	0.691	4.909
Thalidomide Taxotere	(2.50)	0.693	0.003	0.690	4.896
PSA-TRICOM Post	(2.10)	0.693	0.008	0.685	4.842
Thalidomide Post	(2.10)	0.693	0.008	0.685	4.842
PSA-TRICOM Pre	(2.00)	0.693	0.010	0.683	4.819
ATTP Pre	(1.80)	0.693	0.016	0.677	4.755
Thalidomide Pre	(1.80)	0.693	0.016	0.677	4.755
Adesunloye et al 2012					
ATTP	(3.16)	0.693	0.001	0.692	4.924
ARTP	(2.84)	0.693	0.001	0.692	4.915
ATTP [bevacizumab + thalidomide + docetaxel + prednisone],					
ARTP [bevacizumab + lenalidomide + docetaxel + prednisone]					

Surprisingly the time to double is very similar. It is just short of five days. Now these numbers are for patients already diagnosed with PCa. Thus we have a small sample of a measurement which is reflective and cells status which is unknown. Yet that is the best we currently have.

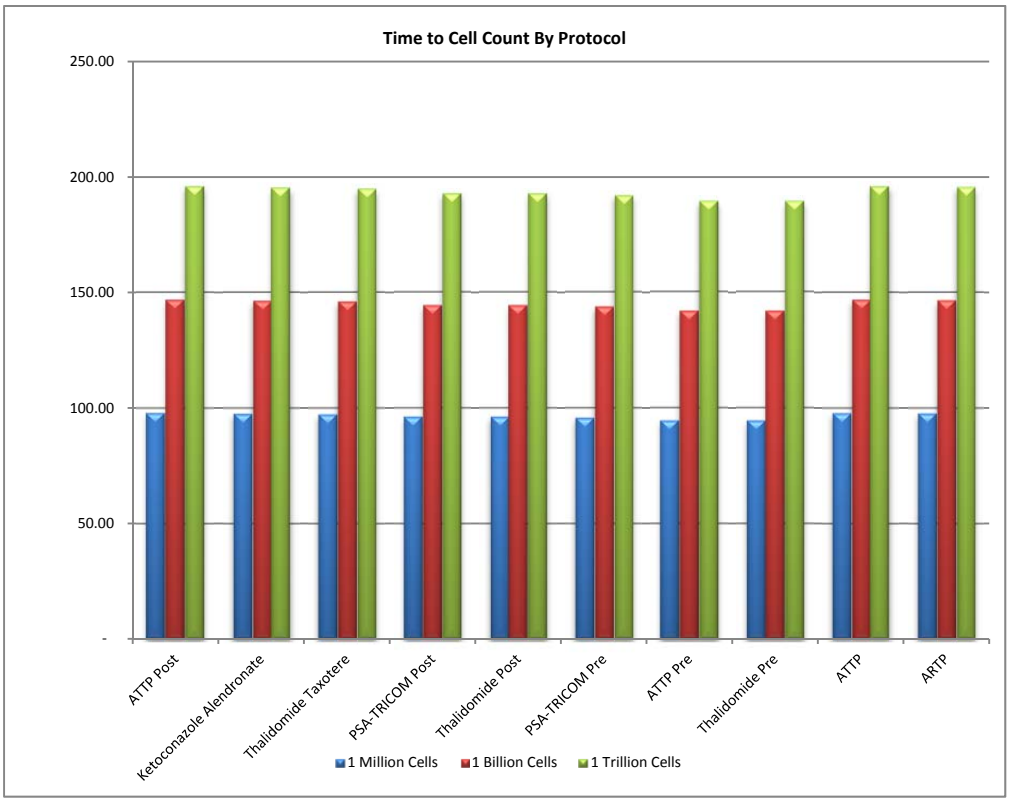
Now we plot this below for the 2010 data.



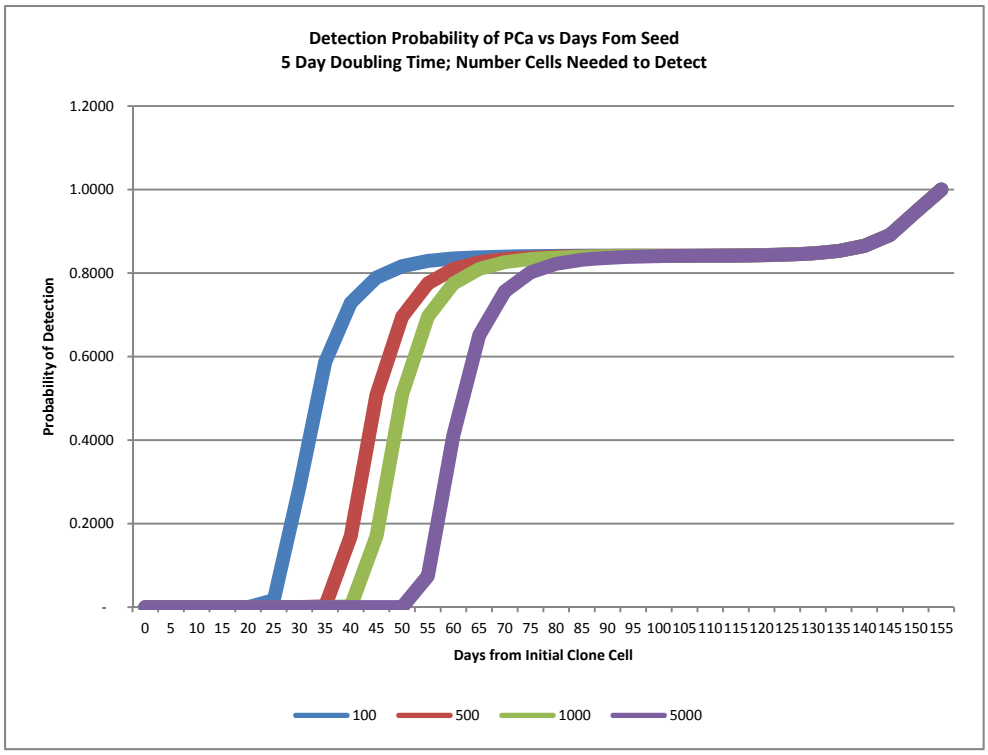
In a similar manner we do the same for the 2012 data below.



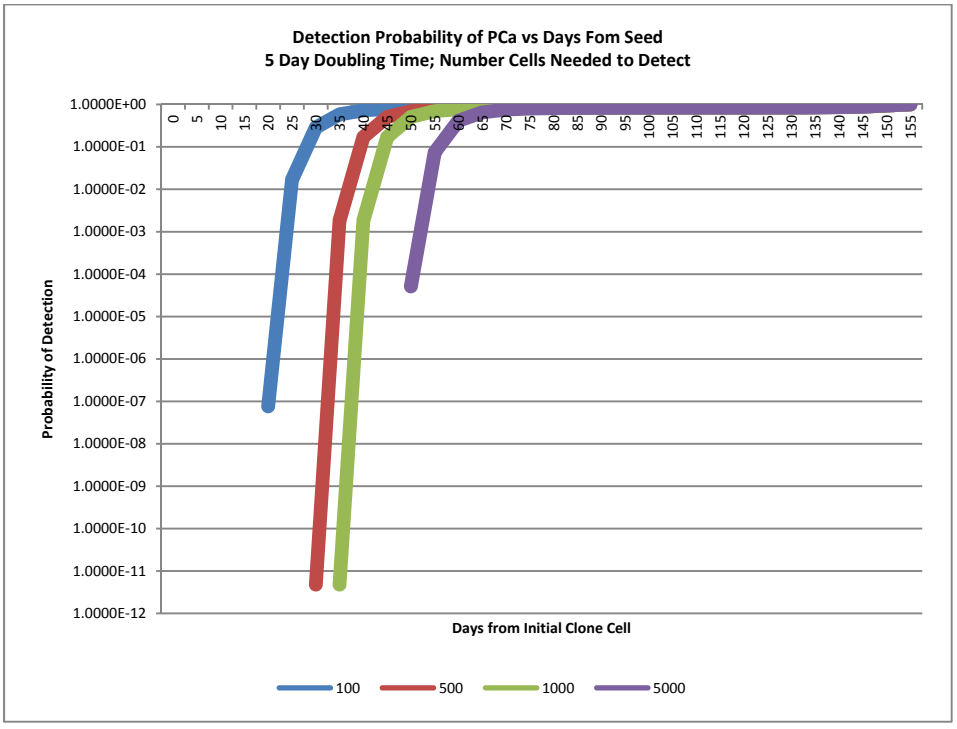
Again for an existing cancer both pre and post treatment numbers show somewhat close doubling times.



Now we ask the question; what is the detection probability using a standard 24 core biopsy to determine the presence of cancer cells as a function of the number of days from incidence. Here we use the time to double numbers from above, start with a binomial assumption, converting to a normal distribution and then calculating as a function of the minimal number of aberrant cells needed to detect.



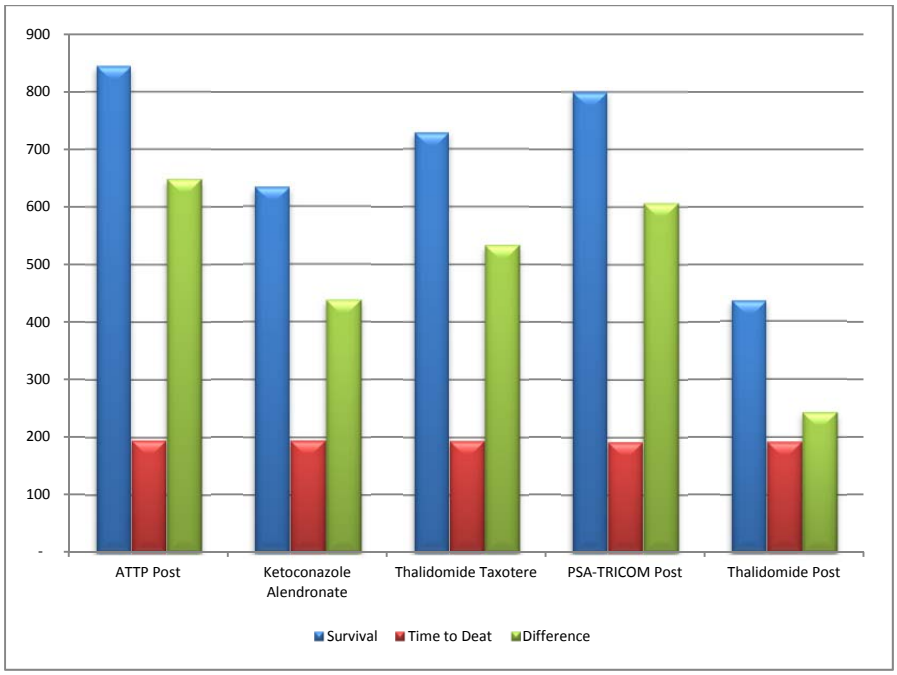
or it can be drawn on a logarithmic scale as follows:



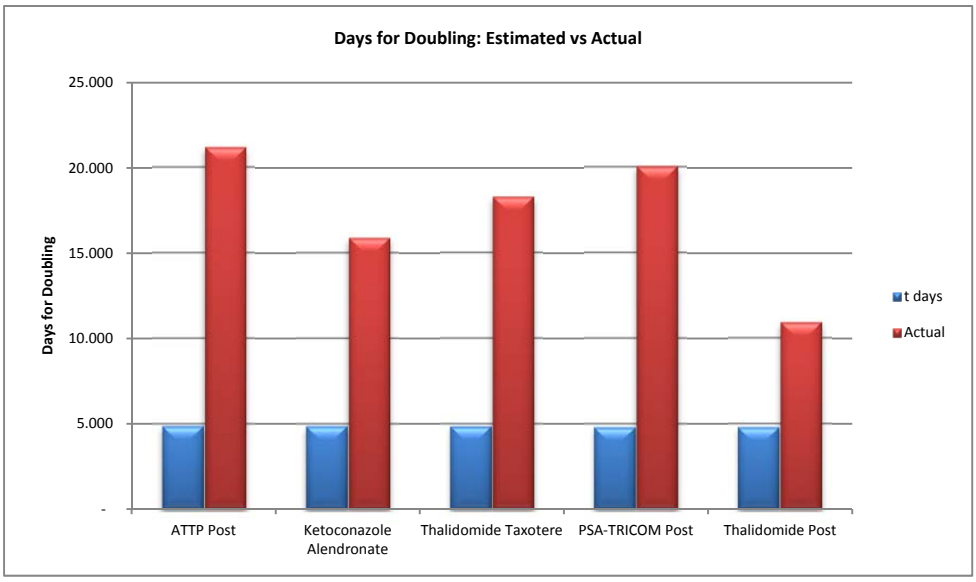
Note in the above that we generally have a low probability to detect but that as the cells grow and proliferate that detection increases significantly. Also note that if one samples early on the

detection probability is low and then suddenly increases making a second biopsy mandatory. A corollary to this is the fact that if we see a problem such as HGPIN the first time and we do not the second, then the proliferation must have disappeared.

The following chart depicts some of the data again. We use the actual time to death. It grossly exceeds a theoretic time to death based upon a cell count of 10^{12}. It is not possible to clarify this discrepancy.



We examined this in several ways. Below we show the days to double based upon the data and then based upon the mortality. Again there is a significant difference which we cannot explain.



Notwithstanding the variance in the numbers, the approach has substantial merit. What it states is that there is measured evidence of doubling times of cancer cells and that using this data one can attain much higher accuracy of biopsy detection and actually seek to quantify it. Our simple binomial to Normal distribution analysis does have some weakness since the cells are not truly randomly distributed but has a clustering effect. This could cause a bias and thus the results may be more confident that what would be the real case. The development of an analysis of this type would have to deal with the clustering or stickiness statistics, which at this time would be highly speculative.

10 OBSERVATIONS

We have used this report as a vehicle to examine the elements of the proposed cancer dynamics model. The basic principles of the model should be reiterated. Specifically:

1. This is not a bench related model.

2. The model is phrased to ask for input values in a more global manner than is usual performed. The following are specific inputs:

a. What are the transition probabilities for the genetic changes observed in cancers. The Gundem model is a powerful and compelling window to those types of changes. The abstract pure clonal model is not sine qua non.

b. What are the parameters for the dynamics. For example, what are the proliferation constants and how do they change by environment and by gene makeup. What are the diffusion constants and how can they best be described.

c. Is the unit of cells per unit volume the key metric?

d. Where do we encounter a stem cell in this model, if at all?

e. How do we deal with cell to cell signalling or is included in the gross parameters.

3. The development of this model is akin to that in electrical engineering versus physics. As an engineering model we deal with an abstraction called resistance and current, voltage and inductance, watts and capacitance. Yes, they can be developed in a detailed manner of the physicist, but their practical application is via abstract generalizations.

One of the areas that has not been addressed is the impact of the immune system on the metastatic behavior. We know that it is significant but there has been no modelling of its effects. That may be a profound weakness yet to be determined.

This study is in no way and end in itself. The purpose was to propose an alternative view to examining cancers using methods which examine gross characteristics using somewhat generalized metrics. We have demonstrated what some of the results using those metrics could be. However, the major observation that can result from this approach is to examine new ways to measure tumor progression. Namely we would propose to examine the dynamics of gene mutations. It is now evident that multiple mutations can occur in different parts of the body from the same initial tumor. Also we can note that there most likely is no single clonal cell. there may be multiple. Obtaining details on the multiplicity of these is critical.

Then also is the study of gross characteristics such as proliferation, diffusion and flow. On proliferation we have used what limited data there is but studies focusing on these are essential. It is also essential to have in vivo proliferation characteristics by gene expression state not just the gross characteristics we show herein. Diffusion is an as yet examined process. We know that

cells lose their local adhesive ability when they become malignant, a key characteristic. Then what causes their movement and at what rate. Is malignant cell diffusion grossly driven by cell density or is there some other mechanism? Can we use a simple diffusion model? What are the diffusion constants by gene expression state?

Finally we have the questions on flow. Intravasation and extravasation is a complex process as we have discussed. Yet at the simplest level it is a flow from local to distant. How the flow really functions may be critical to therapeutic efforts but may be irrelevant to the gross process dynamics. We seek only the gross flow rate.

Overall this is still an early work in progress.

11 APPENDIX: CALCULATION OF PROBABILITIES

We want to provide the methodology used to calculate the probabilities. We assume a growth rate using a doubling time. Thus if we assume the following:

1. A defined doubling time

2. A total organ cell size.

3. Time in days from the initial mutation to the current.

3. The total mutated cell at some time t in days

4. The ratio of mutated cells to benign cells, assuming benign are constant.

5. That the cells do not cluster but are randomly distributed. This is generally not correct but can be considered a second order effect. We shall deal with this latter.

Them we have:

$$N_{\text{mutants}}(t) = 2^T$$
$$where$$
$$T = \frac{t}{T_{double}}$$

Then we can define binomial probabilities as follows:

$$p = \frac{N_{\text{mutant}}}{N_{Total}} = \frac{N_{\text{mutant}}}{N_{benign} + N_{\text{mutant}}}$$
$$q = 1 - p$$

Now we can assume a Normal approximation to the binomial since we have a large sample so we have:

$$m = Np$$
$$\sigma^2 = Np(1-p)$$
$$f(x) = \frac{1}{\sqrt{2\pi\sigma^2}} \exp(-\frac{1}{2\sigma^2}(x-m)^2)$$

From which we can determine the probability that for a certain limit we will have a desired cell or number of cells. Namely we want the probability that we have M or more.

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