THYROID CANCER: SEEK, AND YE SHALL FIND

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There is a dramatic increase in diagnosed thyroid cancers per population but no significant change in mortality per population. This report examines thyroid cancers and looks at them as an interesting target to examine such constructs as the cancer stem cell, EMT mechanism, immunotherapeutics, miRNAs and other challenging topics.

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Contents

1 INTRODUCTION

Thyroid cancer has seen an explosion in incidence but no significant increase in mortality. Like prostate and breast cancers many of them are found in a putatively indolent form. The question is; are we looking more and thus finding more, or is the "more" we find different than those we found in days gone by[1]. The more we are able to look the more we find things that appear suspicious yet these things we find may have had little consequence in year back. Watchful waiting seems to be the slogan for men and their prostates, unlike the cut anywhere for women. How does this apply to the thyroid? For example, the mortality rate per 100,000 people has remained almost constant whereas incidence per 100,000 people has increased. Deceptively this means the mortality per patient has decreased, yet the actual mortality per person is constant. This means that with a certain class of thyroid cancer we have made little progress but we have been able to identify more.

Trees and humans have more in common than we think. Old trees get infected with viruses and get large tumors. Yet these tumors do not metastasize. Old humans get the same, but some of these metastasize. The corollary is that both trees and humans as they age have clusters of cells that are not normal, that could be considered a malignancy. Humans have an immune system that at times is better than trees and this system attacks this neoplasia and rids the system of it. Trees have a much more primitive system, it just encapsulates it in a big burl. The more we can examine the human by our tools the more we may very well find. The prostate is a classic example. Men may very well die in old age with both prostate and thyroid neoplasia. We have examined the prostate elsewhere and we examine the thyroid here[2].

We believe that this is an interesting question given several factors. First the thyroid is involved with both sexes, albeit the incidence much higher in females at younger age. Second, it is a gland near the surface, readily accessible for examination. Third, as a gland it has many of the characteristics of other significant glandular carcinomas. Fourth, pathological studies can be performed less invasively that say the prostate or breast.

As Lim et al have noted recently:

In the United States, thyroid cancer incidence rates have increased by 211% between 1975 and 2013, with papillary thyroid cancer (PTC), the most common and least aggressive histologic type, accounting for most of the new cases. Some investigators have suggested that overdiagnosis, or the increased ability to detect and diagnose small indolent tumors that would never otherwise cause symptoms or require treatment, explains a substantial proportion of the increase.

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[1] Matthew 7:7-8 King James Version (KJV): 7 Ask, and it shall be given you; seek, and ye shall find; knock, and it shall be opened unto you: 8 For every one that asketh receiveth; and he that seeketh findeth; and to him that knocketh it shall be opened.

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

However, growing evidence supports a true increase in the occurrence of thyroid cancer. An analysis of Surveillance, Epidemiology, and End Results(SEER) cancer registry data from 1980-2005 revealed substantial increases in the incidence of advanced-stage PTCs and PTCs greater than 5 cm in diameter; these tumors are generally large enough to be detected via palpation, to cause symptoms, or both. The rates of increase for the largest (>5cm) and the smallest PTCs(≤1 cm) were nearly equal among white women, a group considered to be particularly susceptible to overdiagnosis.

In contrast Welch and Doherty have also noted:

This year, more than 50,000 people in the United States will be diagnosed with thyroid cancer. Three quarters of these diagnoses will be in women; their median age at diagnosis will be about 50 years (for context, the median age at the time of breast cancer diagnosis is 62). Thyroid cancer has become an increasingly common diagnosis for Americans: over the past 25 years, its incidence has tripled — largely reflecting the detection of small papillary thyroid cancers. Despite this dramatic rise in incidence, mortality due to thyroid cancer has remained stable, which suggests that there is widespread overdiagnosis — detection of disease that is not destined to cause clinical illness or death.

Consequently, efforts to reduce thyroid cancer detection are clearly warranted — for example, refraining from screening for cancers and from biopsying small thyroid nodules, as advocated by the American Thyroid Association. Also needed, however, are efforts to reduce overtreatment. We support the option of active surveillance for selected patients with small papillary thyroid cancers, but we recognize that some patients will prefer to have their cancer removed. In such cases, the question becomes how much thyroid to resect. The "complete" operation for thyroid cancer is total thyroidectomy. It carries a risk of injury to either recurrent laryngeal nerve (or, rarely, both of them) and a risk of hypoparathyroidism due to damage to all four parathyroid glands; it also necessitates lifelong thyroid hormone replacement. The less extensive operation is thyroid lobectomy, or removal of about half the thyroid gland.

This surgery carries a lower risk of nerve damage, avoids the risk of hypoparathyroidism altogether, and preserves thyroid tissue — for many patients, obviating the need for permanent thyroid hormone– replacement therapy. Adjuvant therapy with radioactive iodine (RAI) must be preceded by total thyroidectomy.

It has become increasingly clear that the choice between total thyroidectomy (with or without RAI therapy) and lobectomy has little effect on the risk of death from thyroid cancer. ...the 25-year risk of death due to thyroid cancer in patients with localized papillary thyroid cancer (≤2 cm in diameter) treated with either total thyroidectomy or lobectomy. ...

two facts about small papillary thyroid cancers: first, the risk of death from thyroid cancer is extremely low (roughly 2% over 25 years), and second, that risk is unaffected by the choice of procedure. Given the additional harms of total thyroidectomy, one would expect that the recognition of similar effectiveness would lead lobectomy to become the dominant procedure, especially given the increasing detection of small tumors.

But instead, the opposite has happened. ...

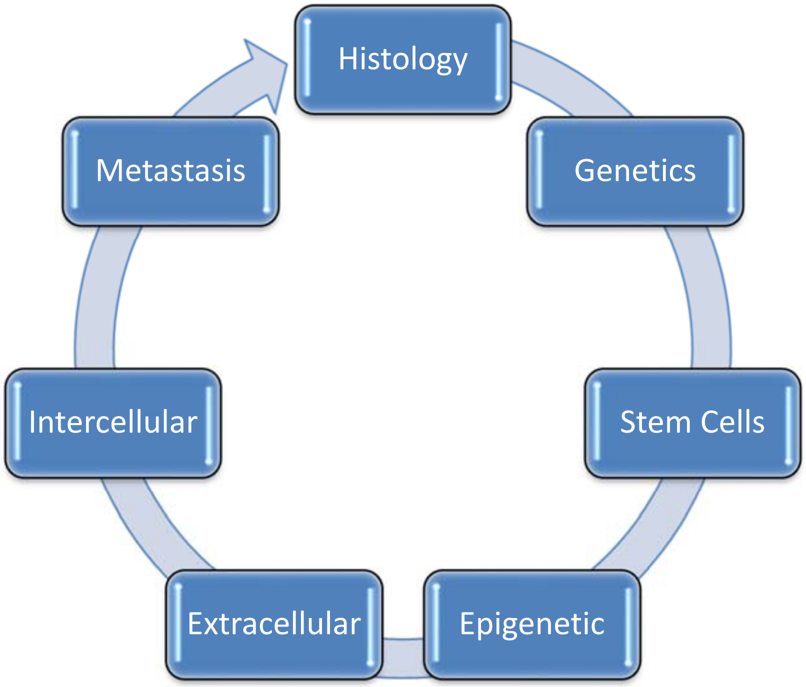
the rate of total thyroidectomy is, in fact, accelerating faster than the rate of lobectomy. Currently, about 80% of patients who have surgery for localized papillary thyroid cancer (≤2 cm in diameter) undergo a total thyroidectomy.

Thus we have an interesting conundrum. A startling increase in such cancers, with the argument that they are real not just histologic artifacts and yet second that no matter how indolent they are they must be totally excised by organ extraction. In many ways this reflects the arguments we have seen in prostate cancer (PCa) over the past decade.

In this paper we examine several areas of this issue. First we examine the various types of thyroid cancers. In many ways they parallel other glandular type tumors but as expected some of the morphology is different. Second we then present some of the recent statistics as we have noted above. This we do in some detail. Then we examine the genomics. As with many cancers today it is often more important to understand the genomic profiles and with Next Gen sequencing this can be accomplished somewhat readily. This does of course raise the issue of the Cancer Stem Cell. We hold this off to the end but it is a critical issue when examining genetic profiles. Namely what cell(s) are we measuring expression on? Next we examine the miRNA issues. Here we argue that miRNAs play a significant role in both expression and in metastasis. Then we focus on the epigenetic issues such as methylation and acetylation. Finally we conclude with several observations. We outline some of these in the following Figure.

Histology	•Shape, orientation, etc
Genomics	•Pathways and blockages •Growth factors, cell stability, vascularization etc
miRNAs	•The internal and external influences, viz exosomes
Epigenetics	•Methylated DNA and methylated and acetylated histones
Stem Cells	•Existens, identification neutralization
Immunotherapy	•Targeting and activation via cell markers using innate and adaptive cells

The issue we try to develop here is that thyroid cancer is an interesting study in the impact of genomics and histology. For decades the assessment of a thyroid tumor has been accomplished by a histological examination. The two general extremes are the papillary form where the shape is lost to the growth of outcropping called papilla and the follicular form where the cells seem just to multiply. Strangely there is a variant called the follicular variant of papillary thyroid cancer, often a small single lesion less than 1 cm which may very well be indolent. Yet unlike such other indolent neoplasia this is still labelled a T1a carcinoma. The issue being that it has the potential to metastasize. The figure below depicts the logic we are attempting to instill on this topic. Namely go from histology thru genomics and eventually back to histology.



There is no one simple answer to many of these issues. Yet there has been a growth of literature exploring each. We currently can employ next generation sequencing, NGS, on thyroid tissues, yet as we have discussed elsewhere this may not tell us a great deal, unless the tumor is extensive. The key issue is the need to determine and examine a stem cell. That mean identifying them and then on a cell by cell basis perform NGS. Yet NGS just allow us to look for what we think to look for. Perhaps we do not know what leads to such factors as epithelial to mesenchymal transitions, EMTs, a key change in a malignancy, and then what of the metastatic behavior of miRNAs and exosomes in communicating with distant cells.

Thus we explore some of these issues in the context of the thyroid, an organ like many other glandular organs yet more accessible than any others.

2 THYROID CANCER TYPES AND HISTOLOGY

Our intent here is not to present a pathologists view but just to highlight some of the pathological histological features and they attempt a nexus to the underlying genetic cause. Examining the histology we can ask such questions as:

1. Why do the cells lose their relational aspects?

2. Why do the cells proliferate?

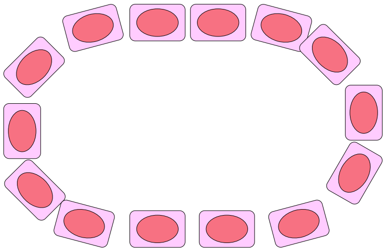
3. What drives vascularization?

4. What are the causes of the morphological changes such as notching and clear nuclei?

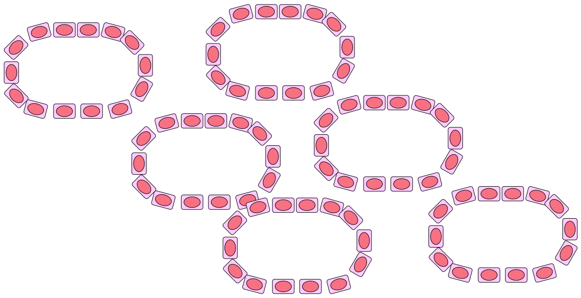
By examining the histology of the cell and especially of the cancerous variants it begs the question; what are the genetic factors which cause or facilitate these changes. Some of these are understood while others remain questionable. This section is in no way an attempt to present the histology of thyroid cancers. It is merely an attempt to raise the question of shape versus cause versus therapeutic.

2.1 THE THYROID CELL

Basically the thyroid cells is the outer side of a thyroid follicle. It is the boundary. This is shown below for a simple thyroid boundary. The cells on the boundary are well behaved and connected. Cell interfaces such as E-cadherin stabilize these cells. Internally to this glandular structure is a collagen internal fill. From this colloid under the pituitary control the T3 and T4 hormones are released. From this is the basis of the thyroid control path.

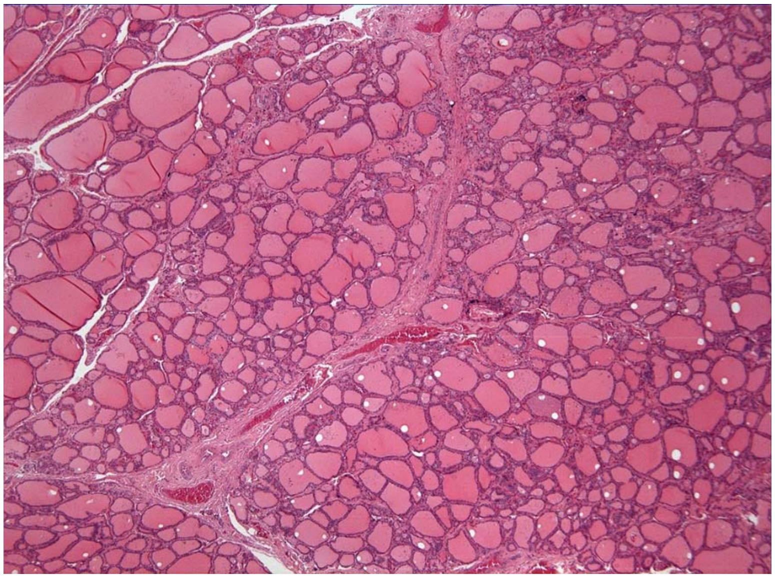


Now the thyroid gland is a compilation of these follicles as shown below. There are blood organs between the follicles and also C cells, cells separate from those that form the effective gland. The highly simplistic view is seen below.



Inside is the collagen material used by the cells to produce T3 and T4.

The normal thyroid cells are shown below (From *Epstein, Biopsy Interpretation of the Thyroid*). The separate cells for enclosures which contain colloid and then it is processed and released by the cell. Surrounding the cell is and there are blood networks throughout the thyroid providing the cells with their requirements and transporting the cell products.



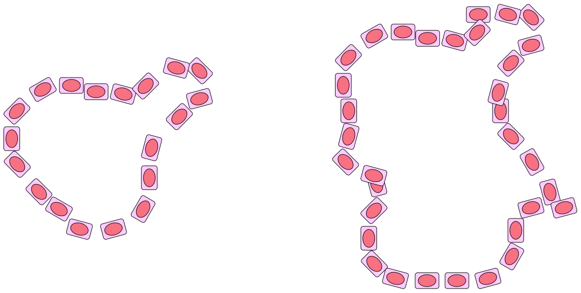
Further specific detail of a follicle is shown below (again from Epstein). Note the clarity and simplicity as well as structure of the cells in each small gland portion:

Note that the cells are well demarcated and organized. Now as we shall see, several variations occur as the cell becomes malignant. Growth results in a proliferation of cells, everywhere, loss of adhesion via EMT results in cell dislocation, and the morphology os individual cells change as well.

2.2 PAPILLARY GROWTH

Now papilla are the small bumps or perturbances of the normal cells which generally are somewhat uniform as we have depicted.

The papillary like cells are shown as below:



The above show the papilla, the bumps or offshoots. It can be argued that this papilla formation is a result of a quasi-EMT process where the E-cadherin bond structure is starting to deteriorate. Namely the genetic control of this is breaking down because of the suppression of the pathways that control epithelial like structure[3]. Now as Nucera and Pontecorvi have noted:

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[3] See McGarty, EMT and Cancers, January 2019, https://www.researchgate.net/publication/330222973_EMT_and_Cancers

Most human thyroid cancers are differentiated papillary carcinomas (PTC). Papillary thyroid microcarcinomas (PTMC) are tumors that measure 1 cm or less. This class of small tumors has proven to be a very common clinical entity in endocrine diseases. PTMC may be present in 30-40% of human autopsies and is often identified incidentally in a thyroid removed for benign clinical nodules.

Although PTMC usually has an excellent long-term prognosis, it can metastasize to neck lymph nodes; however deaths related to this type of thyroid tumor are very rare. Few data exist on molecular pathways that play a role in PTMC development; however, two molecules have been shown to be associated with aggressive PTMC.

S100A4 (calcium-binding protein), which plays a role in angiogenesis, extracellular matrix remodeling, and tumor microenvironment, is over-expressed in metastatic PTMC. In addition, the BRAFV600E mutation, the most common genetic alteration in PTC, is present in many PTMC with extra thyroidal extension and lymph node metastasis.

The above observation is interesting. Namely that almost 40% of people will be harboring small PTCs which unless sampled by a good ultrasound examiner would never be found. Furthermore they would never grow. They also note regarding the papillary growth above:

BRAFV600E triggers a cascade that leads to human papillary thyroid microcarcinoma (PTMC) proliferation. The constitutive kinase activity of BRAFV600E phosphorylates and activates MEK1/2. Phospho-MEK1/2 induces hyperphosphorylation of ERK1/2 which translocates into the nucleus, triggering cell cycle progression, and abnormal cell proliferation by up-regulating cyclins (e.g., Cyclin D1) crucial for the checkpoint machinery in G1-S phases and inhibiting anti-cell cycle cyclins (e.g., p27). Up-regulation of cyclins (e.g., Cyclin D1) leads to hyper-proliferation of papillary thyroid microcarcinoma cells and increase in papillae size.

Now there are several additional and specific histological characteristics. For example, as Das notes[4]:

Psammoma bodies (PBs) are concentric lamellated calcified structures, observed most commonly in papillary thyroid carcinoma (PTC), meningioma, and papillary serous cystadenocarcinoma of ovary but have rarely been reported in other neoplasms and nonneoplastic lesions. PBs are said to represent a process of dystrophic calcification.

Despite numerous ancillary studies over a span of three and half decades, formation of PBs remains a poorly understood mechanism. Ultrastructural study of PTC has shown that thickening of the base lamina in vascular stalk of neoplastic papillae followed by thrombosis, calcification, and tumor cell necrosis leads to formation of PBs. Studies on serous cystadenocarcinoma of ovary and meningioma, however, revealed that collagen production by neoplastic cells and subsequent calcification was responsible for the formation of PBs.

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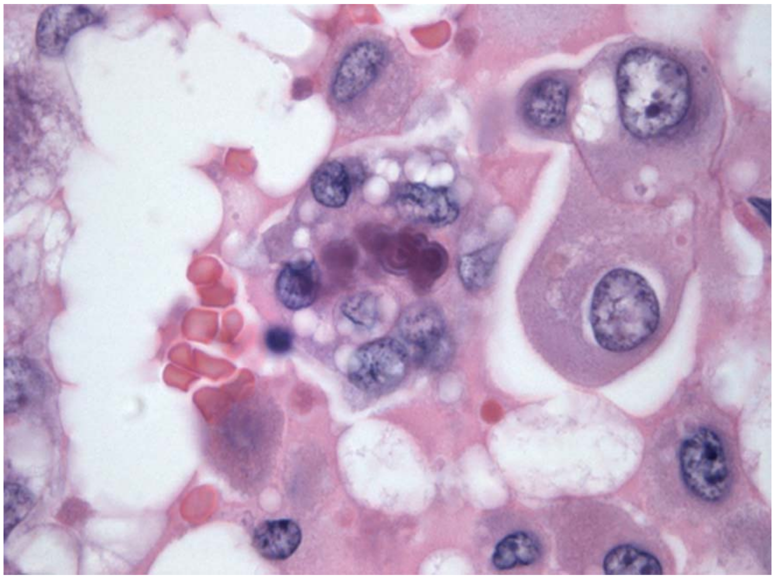
[4] Note: A psammoma body is a round collection of calcium, seen microscopically. The term is derived from the Greek word ψάμμος (psámmos), meaning "sand".

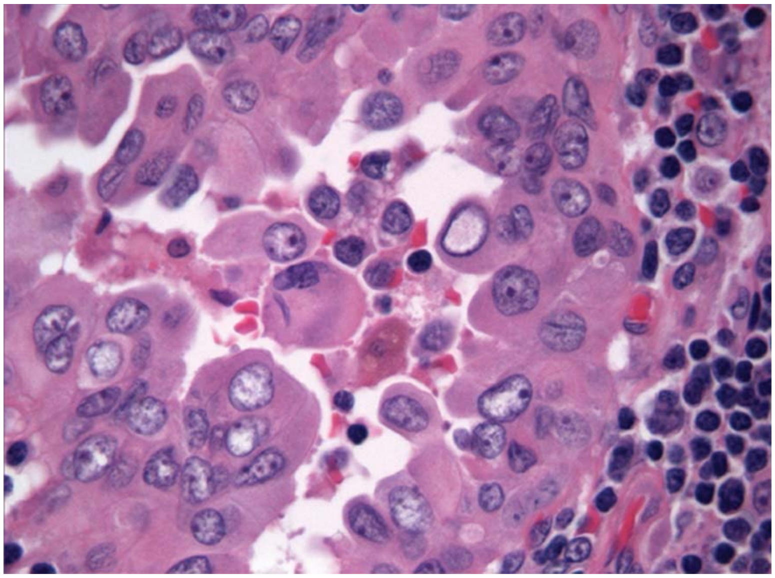
The existence of some precursor forms of PBs was reported in meningiomas and more recently in PTC, which were mostly in the form of extracellular hyaline globules surrounded by well-preserved neoplastic cells or in a smaller number of cases intracytoplasmic bodies liberated from intact tumor cells.

Cellular degeneration and necrosis, leading to the disappearance of neoplastic cells, were noticed by us only around PBs but not around the precursor forms. Based on the above findings, it is suggested that rather than being the outcome of dystrophic calcification of dead or dying tissue, PBs may indeed represent an active biologic process ultimately leading to degeneration/death of tumor cells and retardation of growth of the neoplasm. It may also serve as a barrier against the spread of neoplasm.

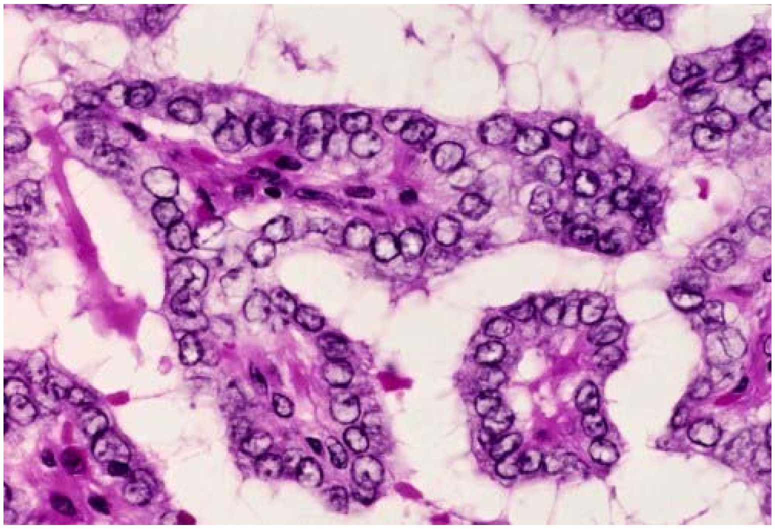
We show psammoma bodies below.



An additional example os specific histological characteristics is one with clear nuclei as shown below:

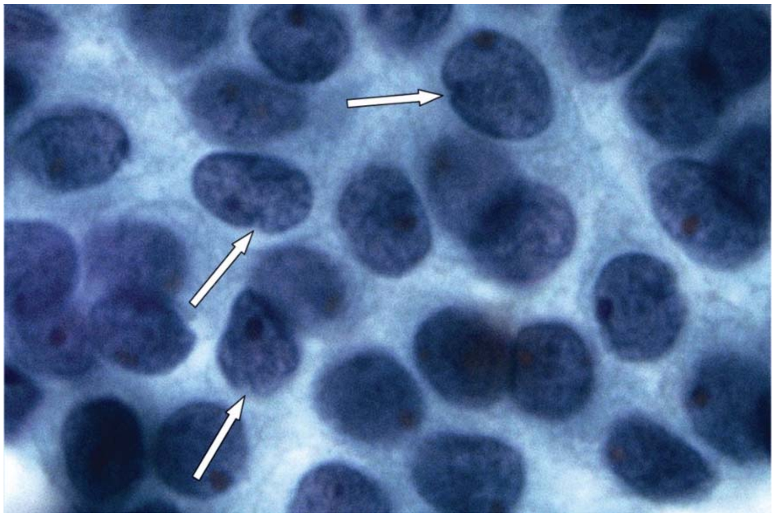


Another example of one type of papillary carcinoma is one with "Orphan Annie" eyes, the wide open white eyes of the nucleus in the cells below.



Note also the well demarcated papillary form with outstretches of the otherwise well-structured cell.

Finally an added one is cells with nuclear grooves is a characteristic that is part of this diagnosis as shown below:



These are small notches seen in the side of the nucleus.

Now these are what a trained histopathologist would be looking for. However, an underlying question is; why are they present and what causes these specific characteristics. We frequently see in medicine the answers to what but not why. Some of these answers are yet to be determined.

2.3 FOLLICULAR GROWTH

In contrast to follicular carcinoma, where the boundary patency gets deformed, follicular carcinoma is where there is a proliferation of the follicular cells.

Baloch and LiVolsi have noted:

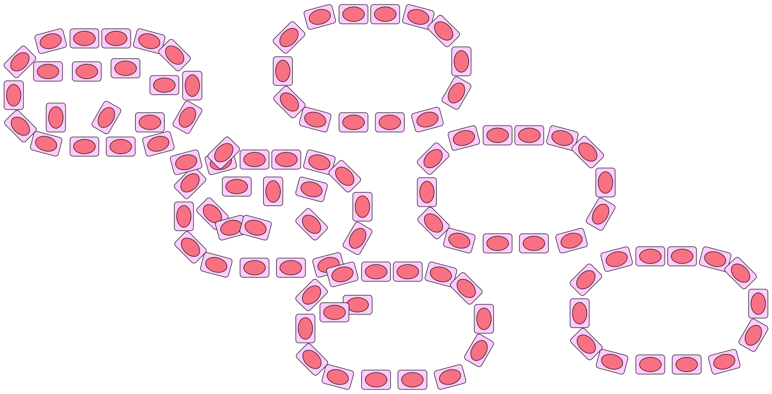
Follicular carcinoma comprises about 5% of thyroid cancers; however, in iodide-deficient areas, this tumor is more prevalent making up 25-40% of thyroid cancers. The true incidence of follicular carcinoma is difficult to determine since the follicular variant of papillary carcinoma may still be placed into this category. Risk factors include iodine deficiency, older age, female gender, and radiation exposure (although the relationship of radiation to follicular carcinoma is far less strong than with papillary cancer).

Clinically, follicular carcinoma usually presents as a solitary mass in the thyroid. Follicular carcinoma has a marked propensity for vascular invasion and avoids lymphatics; hence, true embolic lymph node metastases are exceedingly rare. Follicular carcinoma disseminates hematogenously and metastasizes to bone, lungs, brain, and liver …

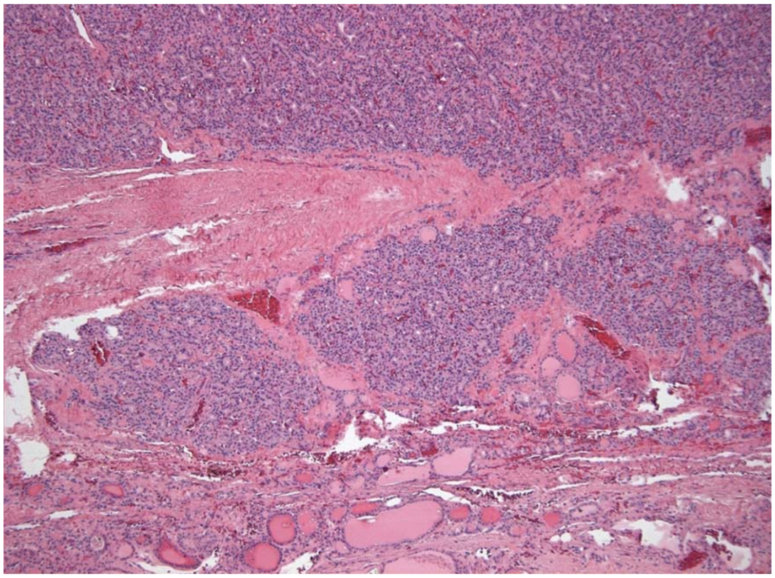
What are the minimum criteria for making this diagnosis? Invasion of the capsule, invasion through the capsule, and invasion into veins in or beyond the capsule represent the diagnostic criteria for carcinoma in a follicular thyroid neoplasm. The criterion for vascular invasion applies solely and strictly to veins in or beyond the capsule, whereas, the definition of capsular invasion is controversial. Some authors require penetration of the capsule to diagnose a *follicular tumor as carcinoma, while others need tumor invasion through the capsule into the surrounding normal thyroid.*

Is capsular invasion insufficient for the diagnosis of follicular cancer? Distant metastases have been reported in follicular carcinoma diagnosed only on the basis of capsular and not vascular invasion, however, in some cases, metastases were already present at initial diagnosis. The presence of vascular invasion is also indicative of malignancy in a follicular tumor. Invasion of vessels within or beyond the lesional capsule is necessary for a definitive diagnosis of vascular invasion. The lesions with vascular invasion should be separated from the minimally invasive follicular carcinomas that show capsular invasion only, because angio-invasive lesions have a greater probability of recurrence and metastasis.

Thus a simplistic view of a follicular cancer is shown below.



We depict a follicular cancer below:



This shows the multiplicity of cells in what was initially a well ordered cell structure filled with collagen.

Note the extensive infiltration. Again in simplistic terms, papillary is a form where we lose shape, namely a putative EMT transition and follicular is where we see extensive proliferation. Clearly both forms may occur.

2.4 NEUROENDOCRINE GROWTH AND MEDULLARY THYROID CANCER

Medullary thyroid cancer is basically a neuroendocrine cancer. Neuroendocrine cancers are an interesting subset of many cancers and it worth reviewing the overall paradigm of their growth.

2.4.1 Neuroendocrine Paradigm

Namely we look at neuroendocrine type effects and thus it requires a slightly more detailed understanding of the prostate As NCI notes[5]:

Neuroendocrine: Having to do with the interactions between the nervous system and the endocrine system. Neuroendocrine describes certain cells that release hormones into the blood in response to stimulation of the nervous system.

We then, in a rationalistic manner, can try and connect the other empirical facts and see if the initial observation can also be logically correct and from that logic ascertain a new therapeutic approach.

A simplistic view of a neuroendocrine system is shown below. Basically the neuro cell activates the endocrine cell which in turn sends out signals to other collections of cells to do whatever they are supposed to do.

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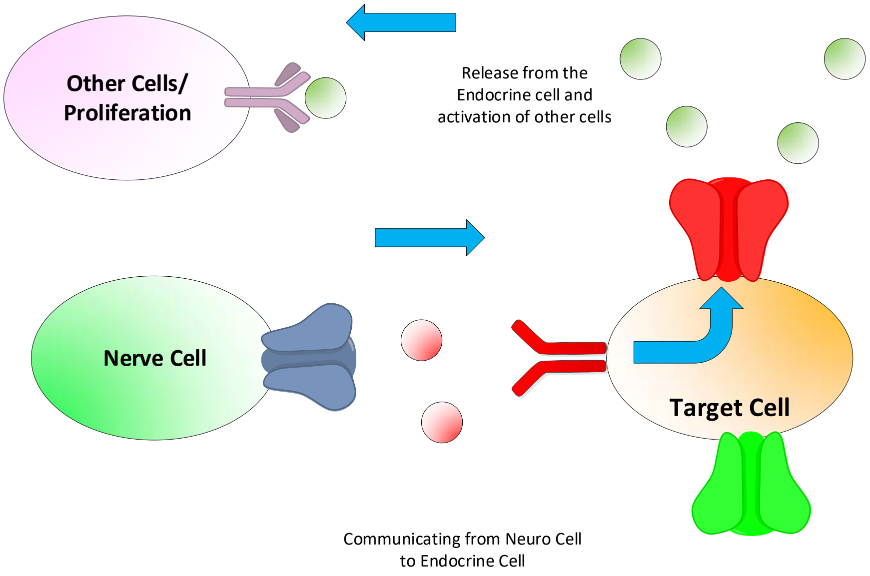
[5] https://www.cancer.gov/publications/dictionaries/cancer-terms/def/neuroendocrine



The above is simplistic but based upon a substantial base of validated cellular signalling factors. Namely these results are empirical in a broad sense. Now when examining various cancers we often look at the cancer cell as being the driving factor. However in a neuroendocrine environment, the cancer cell may be getting its signalling from a cancer initiating cell which in turn is being signaled by a neuro cell. The cancer initiating cell may be blocked by blocking the signalling between it and the causative neuro cell. That is the logical or rationalistic part of this exercise.

The questions now are;

(i) If the malignancy occurs in the neuroendocrine cell, then does it create an environment for proliferation of other cells?

(ii) If the malignancy occurs in the neuroendocrine cell does it send out signals that either block other homeostatic processes or does it accelerate angiogenesis in the new malignancy?

(iii) If the malignancy starts in a non-neuroendocrine cell, are there processes that effectively "turn on" the neuroendocrine cell to facilitate such effects as proliferation, angiogenesis, gene suppression or activation in other cells?

These are but a few of the questions which may be posed. Again we indicate that this is a bit simplistic but it does present the key issues related hereto.

We have examined neuroendocrine driven cancers when examining the prostate. They are simply cancers where a local neuroendocrine cell starts controlling the proliferation process.

As Franz notes:

Medullary thyroid cancer (MTC) is a tumor of the parafollicular C cells that accounts for approximately 10% of all thyroid malignancies. An estimated 75% of MTC cases are sporadic, *and the remaining 25% are familial. Embryologically, these cells originate within the neural crest and function similarly to other neuroendocrine cells within the amine precursor uptake and decarboxylation system. C cells are distributed throughout the entire thyroid gland, although they tend to predominate in the upper poles. Calcitonin, a hormone active in calcium metabolism, is synthesized and secreted by C cells and therefore serves as a useful serum marker for the presence of MTC. Calcitonin levels are most useful in screening individuals who are genetically predisposed to the disease and in following patients who already have been treated. The recent identification of the gene responsible for heritable forms of MTC has allowed earlier identification of individuals at risk for the disease*

2.4.2 Medullary carcinoma

Kim and Kuo have noted:

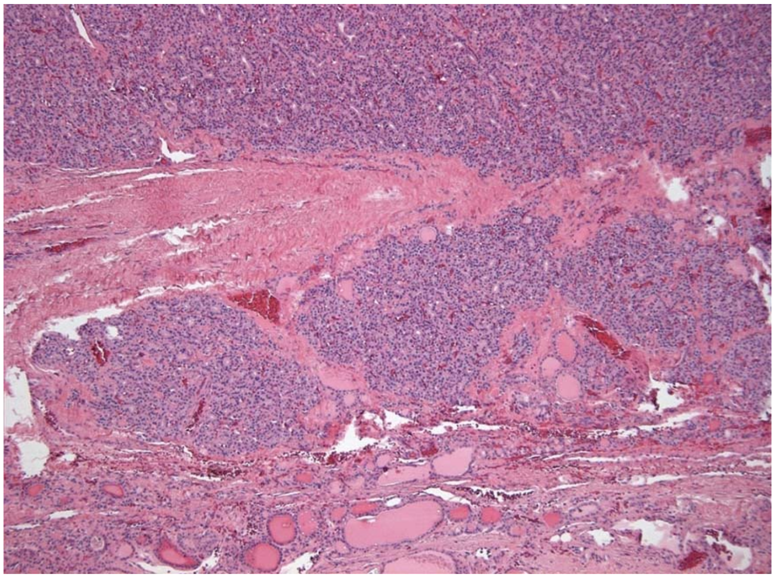
Medullary thyroid carcinoma (MTC) is a rare neuroendocrine tumor derived from the thyroid C cells producing calcitonin. MTC accounts for 0.6% of all thyroid cancers and incidence of MTC increased steadily between 1997 and 2011 in Korea. It occurs either sporadically or in a hereditary form based on germline rearranged during transfection (RET) mutations. MTC can be cured only by complete resection of the thyroid tumor and any loco-regional metastases.

The most appropriate treatment is still less clear in patients with residual or recurrent disease after initial surgery or those with distant metastases because most patients even with metastatic disease have indolent courses with slow progression for several years and MTC is not responsive to either radioactive iodine therapy or thyroid-stimulating hormone suppression. Recently, two tyrosine kinase inhibitors (TKIs), vandetanib and cabozantinib, are approved for use in patients with advanced, metastatic or progressive MTC.

Baloch and LiVolsi note:

Medullary thyroid carcinoma comprises less than 10% of all thyroid malignancies. This tumor is of great diagnostic importance because of its aggressiveness, its close association with multiple endocrine neoplasia syndromes (MEN2A and 2B), and a relationship to a C cell hyperplasia, a probable pre cursor lesion.

While the majority of medullary carcinomas are sporadic, about 10-20% are familial. Since these familial cases have been identified, a gene associated with medullary carcinoma has been identified on chromosome 10 and involves mutations in the RET oncogene.



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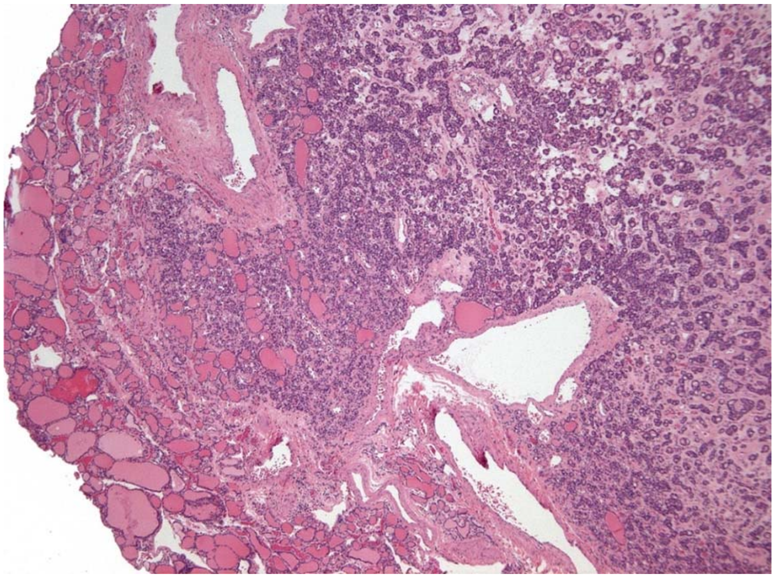


2.5 ANAPLASTIC

This is a highly aggressive cancer with nearly 100% mortality in 6 to 12 months. It is also quite rare but seems to be a sequella to a Graves diseased thyroid. Given its rarity and complexity it is worth just a mention.

3 THYROID CANCER STATISTICS

We now examine some of the statistics regarding thyroid cancers. The following Figure uses the SEER database and plots incidence and mortality. Two observations stand out:

1. Incidence is exploding over the past decades.

2. Mortality has not changed.

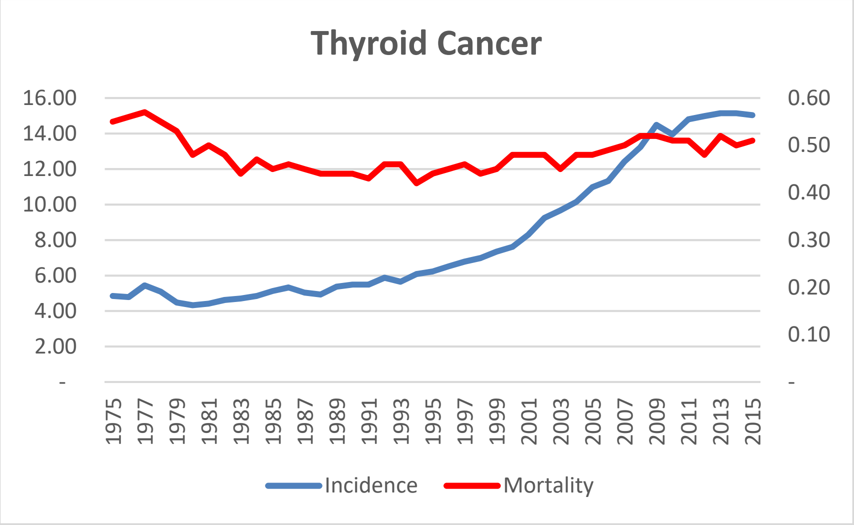
Thus there can be several conclusions:

1. There truly is a massive increase in this type of cancer but we are getting so good at treating it that no one seems to succumb.

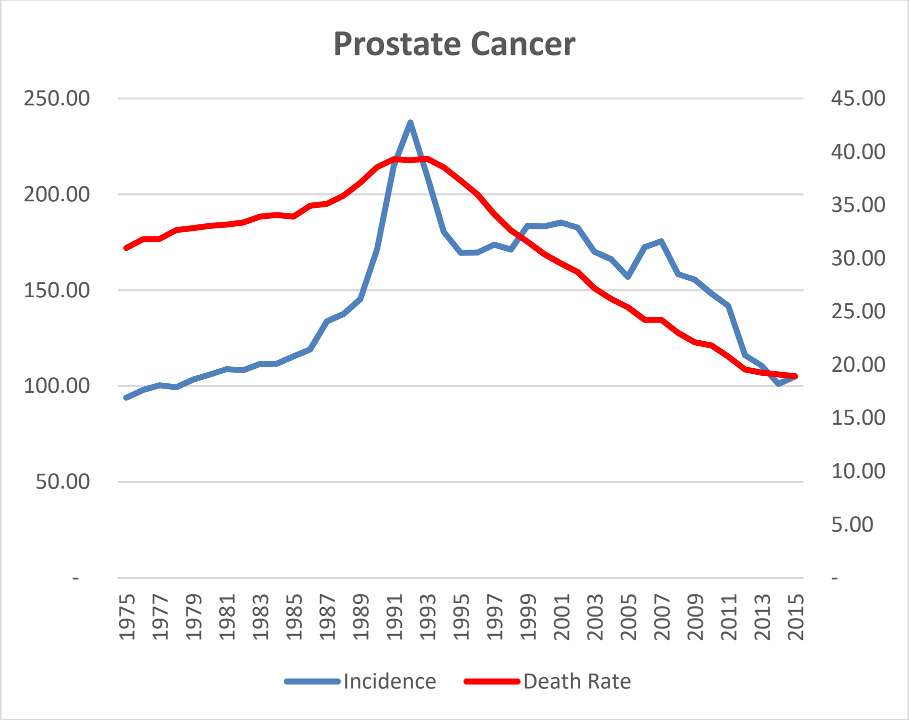
2. We are able to identify these cancers so early that we are getting them and keeping mortality low.

3. What we are calling thyroid cancers would not be called cancer if it were in other organs such as DCIS in the breast, MIS melanoma, or HGPIN in the prostate. Yet in the last three we do know that progression may occur.

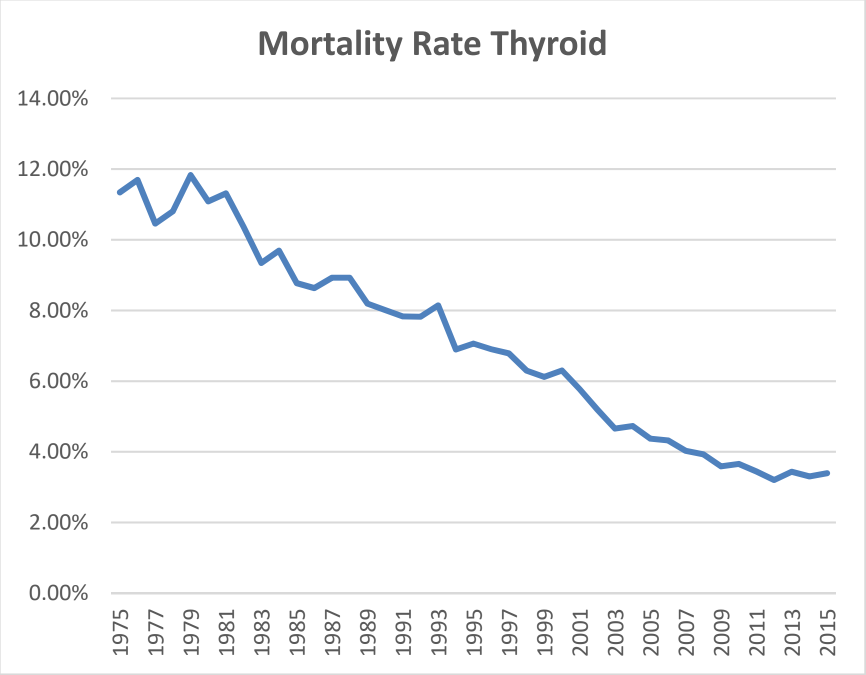
4. The newer ones being identified are not cancer at all but just a hyperplasia because they tend to be confined and have no tendency to metastasize.



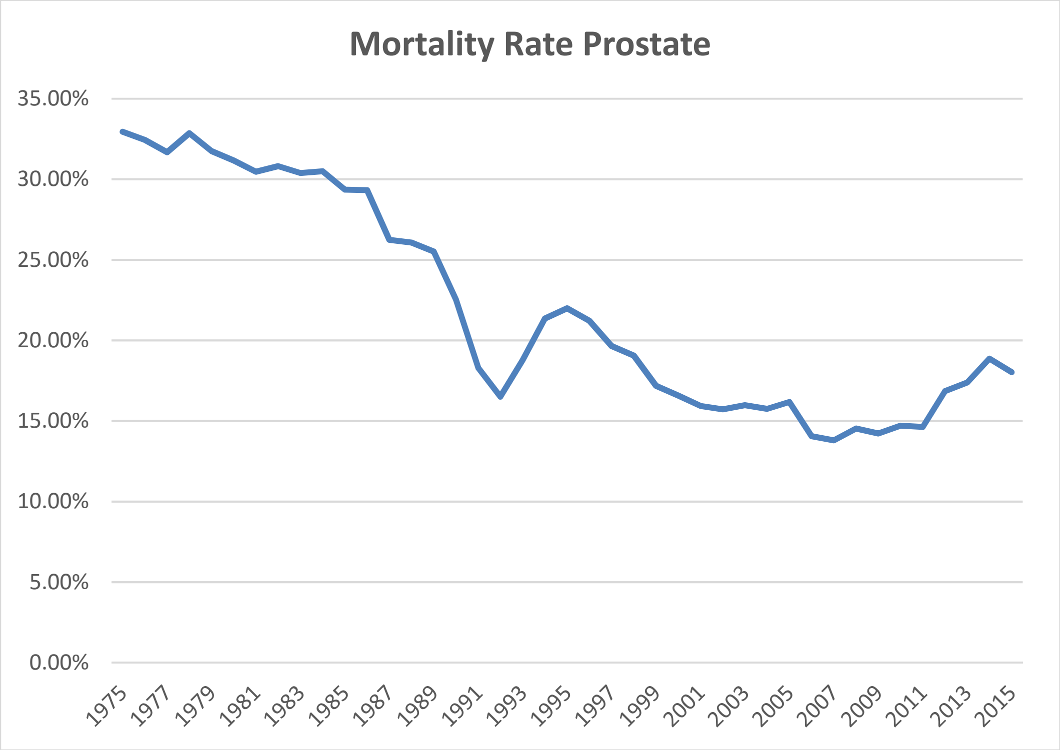
Thus we ask just what is happening? let's look at the prostate model in the Figure below. Same data source and same curves plotted but now for PCa.



The following Figure depicts the mortality rates for thyroid cancer in toto over the same period. This seems to imply that these cancers are being nearly cured whereas we may really just be finding massive numbers of indolent lesions whose progress may never get to the point of resulting in death. This is in sharp contrast to many other cancers.



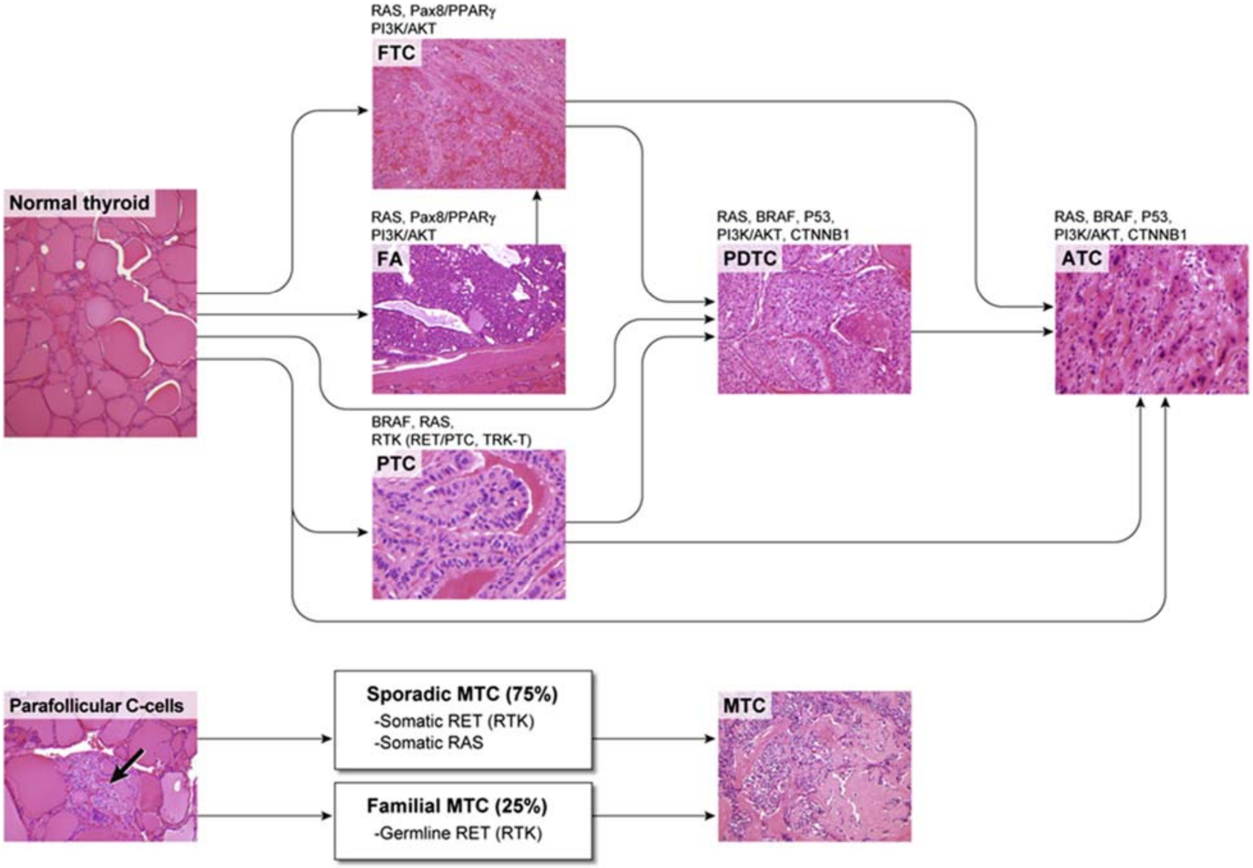
One can compare this to the same curve but for prostate cancer. In the prostate cancer mortality has been cut in half. In the thyroid case it has been reduced by a factor of six. The question is: are both of these artifacts of early detection, and detection of indolent neoplasia?



4 GENOMICS

Over the past two decades the details of the genomics of many cancers have been developed. In the case of thyroid cancer we now have a substantial base.

From Wells and Santoro we have the chart below which depicts the genetic disturbances for various thyroid cancers and their genetic markers.



We will now explore several genes which are alleged to play a role in thyroid cancers. We first look at those examined in fusion panels and then those examined for somatic mutations. The Table below is from Nikiforov (2020) and presents the genetic profiles of various types of thyroid malignancies.

			FA	HCA	NIFTP	FTC	HCC	PTC	PDTC	ATC	MTC[a]
Point mutations and indels		*BRAF* V600E, %					0	40-45	5-30	10-45	<5
		RAS, %	20-30	10-20	30-40	40-50	10-20	20	20-40	20-40	10-15
		EIF1AX, %	10-20	10-15	5-10	10-15	10-15	<5	10	10	
		PTEN, %	10-15		-5	10-15	10-15	<3	5-20	10-15	
		DICER1, %	10-15		-5	10-15		<5			
		7P53, %				<10	15-20	<5	10-30	50-70	<5
		TERT, %				15	10-20	5-10	30-50	70	
		PIK3CA, %	<5			<5		<5	5-20	5-18	
		AKT1, %							<5	<5	
		RET, %									40-50
Large-scale alterations	Fusions	*RET/PTC*, %						5-10	<5	<1	
		PPARG, %	5-10		20-30	10-20		<5	5-7	<1	
		NTRK1/3, %						<5	1-5		
		ALK, %						<5	5-10	<5	2
		THADA, %	5-10		20-30	<5		5			
		Somatic copy number alterations, %	20-40	40-50	20	40-50	70	5-10	50	90	20

NOTE: ATC, anaplastic thyroid carcinoma; FA, follicular adenoma; FTC, follicular thyroid carcinoma; HCA, Hürthle cell adenoma; HCC, Hürthle cell carcinoma; MTC, medullary thyroid carcinoma; NIFTP, noninvasive follicular thyroid neoplasm with papillary-like nuclear features; PDTC, poorly differentiated thyroid carcinoma; PTC, papillary thyroid carcinoma. aSporadic MTC.

4.1 FUSION PANEL

Some of the common gene fusions examined for thyroid cancer are listed in the Table below.

Gene	***Function***
ALK[6]	This gene encodes a receptor tyrosine kinase, which belongs to the insulin receptor superfamily. This protein comprises an extracellular domain, an hydrophobic stretch corresponding to a single pass transmembrane region, and an intracellular kinase domain. It plays an important role in the development of the brain and exerts its effects on specific neurons in the nervous system. This gene has been found to be rearranged, mutated, or amplified in a series of tumours including anaplastic large cell lymphomas, neuroblastoma, and non-small cell lung cancer.

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[6] https://www.ncbi.nlm.nih.gov/gene/238

Gene	***Function***
AXL[7]	The protein encoded by this gene is a member of the Tyro3-Axl-Mer (TAM) receptor tyrosine kinase subfamily. The encoded protein possesses an extracellular domain which is composed of two immunoglobulin-like motifs at the N-terminal, followed by two fibronectin type-III motifs. It transduces signals from the extracellular matrix into the cytoplasm by binding to the vitamin K-dependent protein growth arrest-specific 6 (Gas6). This gene may be involved in several cellular functions including growth, migration, aggregation and anti-inflammation in multiple cell types.
BRAF[8]	This gene encodes a protein belonging to the RAF family of serine/threonine protein kinases. This protein plays a role in regulating the MAP kinase/ERK signaling pathway, which affects cell division, differentiation, and secretion. Mutations in this gene, most commonly the V600E mutation, are the most frequently identified cancer-causing mutations in melanoma, and have been identified in various other cancers as well, including non-Hodgkin lymphoma, colorectal cancer, thyroid carcinoma, non-small cell lung carcinoma, hairy cell leukemia and adenocarcinoma of lung.
EGFR[9]	The protein encoded by this gene is a transmembrane glycoprotein that is a member of the protein kinase superfamily. This protein is a receptor for members of the epidermal growth factor family. EGFR is a cell surface protein that binds to epidermal growth factor. Binding of the protein to a ligand induces receptor dimerization and tyrosine autophosphorylation and leads to cell proliferation. Mutations in this gene are associated with lung cancer.
FGFR1[10]	The protein encoded by this gene is a member of the fibroblast growth factor receptor (FGFR) family, where amino acid sequence is highly conserved between members and throughout evolution. FGFR family members differ from one another in their ligand affinities and tissue distribution. A full-length representative protein consists of an extracellular region, composed of three immunoglobulin-like domains, a single hydrophobic membrane-spanning segment and a cytoplasmic tyrosine kinase domain. The extracellular portion of the protein interacts with fibroblast growth factors, setting in motion a cascade of downstream signals, ultimately influencing mitogenesis and differentiation. This particular family member binds both acidic and basic fibroblast growth factors and is involved in limb induction.

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[7] https://www.ncbi.nlm.nih.gov/gene/558

[8] https://www.ncbi.nlm.nih.gov/gene/673

[9] https://www.ncbi.nlm.nih.gov/gene/1956

[10] https://www.ncbi.nlm.nih.gov/gene/2260

Gene	***Function***
FGFR2[11]	The protein encoded by this gene is a member of the fibroblast growth factor receptor family, where amino acid sequence is highly conserved between members and throughout evolution. FGFR family members differ from one another in their ligand affinities and tissue distribution. A full-length representative protein consists of an extracellular region, composed of three immunoglobulin-like domains, a single hydrophobic membrane-spanning segment and a cytoplasmic tyrosine kinase domain. The extracellular portion of the protein interacts with fibroblast growth factors, setting in motion a cascade of downstream signals, ultimately influencing mitogenesis and differentiation. This particular family member is a high-affinity receptor for acidic, basic and/or keratinocyte growth factor, depending on the isoform.
FGFR3[12]	This gene encodes a member of the fibroblast growth factor receptor (FGFR) family, with its amino acid sequence being highly conserved between members and among divergent species. FGFR family members differ from one another in their ligand affinities and tissue distribution. A full-length representative protein would consist of an extracellular region, composed of three immunoglobulin-like domains, a single hydrophobic membrane-spanning segment and a cytoplasmic tyrosine kinase domain. The extracellular portion of the protein interacts with fibroblast growth factors, setting in motion a cascade of downstream signals, ultimately influencing mitogenesis and differentiation.
MET[13],[14]	This gene encodes a member of the receptor tyrosine kinase family of proteins and the product of the proto-oncogene MET. The encoded preproprotein is proteolytically processed to generate alpha and beta subunits that are linked via disulfide bonds to form the mature receptor. Further processing of the beta subunit results in the formation of the M10 peptide, which has been shown to reduce lung fibrosis. Binding of its ligand, hepatocyte growth factor, induces dimerization and activation of the receptor, which plays a role in cellular survival, embryogenesis, and cellular migration and invasion.
MYB[15]	This gene encodes a protein with three HTH DNA-binding domains that functions as a transcription regulator. This protein plays an essential role in the regulation of hematopoiesis. This gene may be aberrantly expressed or rearranged or undergo translocation in leukemias and lymphomas, and is considered to be an oncogene.
NRG1[16]	The protein encoded by this gene is a membrane glycoprotein that mediates cell-cell signaling and plays a critical role in the growth and development of multiple organ systems. An extraordinary variety of different isoforms are produced from this gene through alternative promoter usage and splicing.

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[11] https://www.ncbi.nlm.nih.gov/gene/2263

[12] https://www.ncbi.nlm.nih.gov/gene/2261

[13] Kenji TAKEUCHI* and Fumiaki ITO

[14] https://www.ncbi.nlm.nih.gov/gene/4233

[15] https://www.ncbi.nlm.nih.gov/gene/4602

[16] https://www.ncbi.nlm.nih.gov/gene/3084

Gene	***Function***
NTRKl[17]	This gene encodes a member of the neurotrophic tyrosine kinase receptor (NTKR) family. This kinase is a membrane-bound receptor that, upon neurotrophin binding, phosphorylates itself and members of the MAPK pathway. The presence of this kinase leads to cell differentiation and may play a role in specifying sensory neuron subtypes. Mutations in this gene have been associated with congenital insensitivity to pain, anhidrosis, self-mutilating behavior, cognitive disability and cancer.
NTRK2[18]	This gene encodes a member of the neurotrophic tyrosine receptor kinase (NTRK) family. This kinase is a membrane-bound receptor that, upon neurotrophin binding, phosphorylates itself and members of the MAPK pathway. Signalling through this kinase leads to cell differentiation. Mutations in this gene have been associated with obesity and mood disorders.
NTRK3[19]	This gene encodes a member of the neurotrophic tyrosine receptor kinase (NTRK) family. This kinase is a membrane-bound receptor that, upon neurotrophin binding, phosphorylates itself and members of the MAPK pathway. Signalling through this kinase leads to cell differentiation and may play a role in the development of proprioceptive neurons that sense body position. Mutations in this gene have been associated with medulloblastomas, secretory breast carcinomas and other cancers. Several transcript variants encoding different isoforms have been found for this gene.
PDGRFA[20]	his gene encodes a cell surface tyrosine kinase receptor for members of the platelet-derived growth factor family. These growth factors are mitogens for cells of mesenchymal origin. The identity of the growth factor bound to a receptor monomer determines whether the functional receptor is a homodimer or a heterodimer, composed of both platelet-derived growth factor receptor alpha and beta polypeptides. Studies suggest that this gene plays a role in organ development, wound healing, and tumor progression. Mutations in this gene have been associated with idiopathic hypereosinophilic syndrome, somatic and familial gastrointestinal stromal tumors, and a variety of other cancers.
PPARG[21]	his gene encodes a member of the peroxisome proliferator-activated receptor (PPAR) subfamily of nuclear receptors. PPARs form heterodimers with retinoid X receptors (RXRs) and these heterodimers regulate transcription of various genes. Three subtypes of PPARs are known: PPAR-alpha, PPAR-delta, and PPAR-gamma. The protein encoded by this gene is PPAR-gamma and is a regulator of adipocyte differentiation. Additionally, PPAR-gamma has been implicated in the pathology of numerous diseases including obesity, diabetes, atherosclerosis and cancer. Alternatively spliced transcript variants that encode different isoforms have been described.

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[17] https://www.ncbi.nlm.nih.gov/gene/4914
[18] https://www.ncbi.nlm.nih.gov/gene/4915
[19] https://www.ncbi.nlm.nih.gov/gene/4916
[20] https://www.ncbi.nlm.nih.gov/gene/5156
[21] https://www.ncbi.nlm.nih.gov/gene/5468

Gene	***Function***
RET[22]	This gene encodes a transmembrane receptor and member of the tyrosine protein kinase family of proteins. Binding of ligands such as GDNF (glial cell-line derived neurotrophic factor) and other related proteins to the encoded receptor stimulates receptor dimerization and activation of downstream signaling pathways that play a role in cell differentiation, growth, migration and survival. The encoded receptor is important in development of the nervous system, and the development of organs and tissues derived from the neural crest. This proto-oncogene can undergo oncogenic activation through both cytogenetic rearrangement and activating point mutations.
ROS1[23]	This proto-oncogene, highly-expressed in a variety of tumor cell lines, belongs to the sevenless subfamily of tyrosine kinase insulin receptor genes. The protein encoded by this gene is a type I integral membrane protein with tyrosine kinase activity. The protein may function as a growth or differentiation factor receptor
THADA[24]	This gene is the target of 2p21 choromosomal aberrations in benign thyroid adenomas. Single nucleotide polymorphisms (SNPs) in this gene may be associated with type 2 diabetes and polycystic ovary syndrome. The encoded protein is likely involved in the death receptor pathway and apoptosis.

4.1.1 ALK

As Murugan and Xing have observed:

Anaplastic lymphoma kinase (ALK) is a member of the insulin receptor subfamily of receptor tyrosine kinases (RTKs), with its encoding gene located on the short arm of chromosome 2.

ALK was initially identified as part of an oncogenic fusion gene, NPM1-ALK (also known as NPMALK), in anaplastic large-cell non–Hodgkin's lymphomas (ALCL). It is also part of the fusion gene EML4-ALK in non–small-cell lung cancer (NSCLC).

There are a few other ALK fusion genes, such as TMP3/4-ALK and RANBP2-ALK, in inflammatory myofibroblastic tumors (IMT). The tyrosine kinase activities of these fusion ALK proteins are aberrantly activated and promote cell proliferation and survival. ALK fusion proteins have also been shown to activate various signaling pathways, among which are the phosphatidylinositol 3-kinase (PI3K)/Akt pathway and the Ras-Raf-MEK- extracellular signal regulated kinase (ERK)/mitogen-activated protein (MAP) kinase pathway with multiple interaction points to mediate the ALK signaling.

4.1.2 EGFR

The epidermal growth factor, EGF, is another GF associated with malignancies. As NCBI notes[25]:

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[22] https://www.ncbi.nlm.nih.gov/gene/5979
[23] https://www.ncbi.nlm.nih.gov/gene/6098
[24] https://www.ncbi.nlm.nih.gov/gene/63892
[25] https://www.ncbi.nlm.nih.gov/gene/1950

This gene encodes a member of the epidermal growth factor superfamily. The encoded preproprotein is proteolytically processed to generate the 53-amino acid epidermal growth factor peptide. This protein acts a potent mitogenic factor that plays an important role in the growth, proliferation and differentiation of numerous cell types. This protein acts by binding with high affinity to the cell surface receptor, epidermal growth factor receptor. Defects in this gene are the cause of hypomagnesemia type 4.

Dysregulation of this gene has been associated with the growth and progression of certain cancers. Alternative splicing results in multiple transcript variants, at least one of which encodes a preproprotein that is proteolytically processed.

4.1.2.1 EGF Functions

We begin with a simple overview of the EGF functions. As Singh et al note:

EGF is the prototypic and founding member of the EGFR ligand family, first identified from submaxillary gland extracts during nerve growth factor studies. The EGF-EGFR ligand-receptor system has greatly enhanced our understanding of receptor tyrosine kinase signaling, as evidenced by more than 70,000 publications for EGF alone. A recent review has distilled our current understanding of EGF and its actions.

More recently, a study uncovered that EGF-induced EGFR signaling enhances production of intracellular reactive oxygen species (ROS) by dual oxidase 1 (DUOX1) This nicely complements earlier studies in which ROS were shown to enhance EGFR signaling by modulating both positive and negative regulators of EGFR signaling (ADAMs and protein tyrosine phosphatases). In another recent study, urinary EGF has been shown to be an independent risk factor for progression of chronic kidney disease, substantiating earlier findings.

They then note its functioning:

Modes of signaling via epidermal growth factor receptor (EGFR) ligands.

Autocrine signaling occurs when a ligand is released from a cell and binds to EGFR on that same cell.

Paracrine signaling refers to the released ligand acting on a nearby cell, usually a different cell type.

Juxtacrine signaling occurs when a non-cleaved, transmembrane ligand binds to EGFR on an adjacent cell; this is best documented for heparin-binding epidermal growth factor-like growth factor (HBEGF). Amphiregulin (AREG), transforming growth factor-alpha (TGFA), and HBEGF, as well as EGFR, can be packaged into signaling competent exosomes. Uptake of exosomal AREG by recipient cells is, at least in part, dependent on EGFR, leading to the term exosomal targeted receptor activation (ExTRAcrine).

ExTRAcrine signaling has features of autocrine, paracrine, and juxtacrine signaling as well as possibly endocrine signaling since EGFR and AREG can be detected in human plasma exosome.

4.1.2.2 EGF and Cancer

Relationships between EGF and cancers are significant. From Yang et al we have the following:

EGF and its receptor (EGFR) have been associated with tumour cell invasion and metastasis initiation.

Dysregulation of EGFR signalling, including receptor over expression and/or activation has been shown to be a significant effector in the progression of human cancers including neoplasms of the brain, lung, breast, ovary, prostate, and pancreas.

A recent study investigated the relationship between EGFR and the adhesion molecule-integrin in human pancreatic carcinoma cells and demonstrated that the crosstalk between EGFR signalling and integrin in the cancer cell membrane is implicated in carcinoma cell invasion and metastasis. Integrins are a family of adhesion proteins that regulate cell migration.

The fact that EGF stimulated integrins-mediated carcinoma cell migration on vitronectin suggests that EGFR regulates cancer cell migration through the adhesion proteins, the integrins. EGFR inhibitors, such as erlotinib, provide clinical benefit in patients with advanced non-small cell lung cancer metastasis which suggests a critical role for EGF and its receptor in the initial steps of cancer metastasis. The mechanism of EGF activation of adhesion proteins in cancer cell remains to be elucidated.

Some studies indicate EGF induces tumour cell invasion and metastasis through de-phosphorylation and downregulation of focal adhesion kinase, while other studies suggest EGFR activates the Src family of kinases (SFK). The fact that activated Src kinase is involved in the rearrangement of the actin cytoskeleton, cell-matrix interactions, and cell-cell adhesion processes that promote cell invasion suggests a role for Src activity in tumour metastasis development.

Added insight is provided by Mendelsohn and Baselga who note:

Human carcinomas frequently express high levels of receptors in the EGF receptor family, and overexpression of at least two of these receptors, the EGF receptor (EGFr) and closely related ErbB2, has been associated with a more aggressive clinical behavior. Further, transfection or activation of high levels of these two receptors in nonmalignant cell lines can lead to a transformed phenotype. For these reasons therapies directed at preventing the function of these receptors have the potential to be useful anti-cancer treatments. In the last two decades monoclonal antibodies (MAbs) which block activation of the EGFr and ErbB2 have been developed.

These MAbs have shown promising preclinical activity and `chimeric' and `humanized' MAbs have been produced in order to obviate the problem of host immune reactions. Clinical activity with these antibodies has been documented: trastuzumab, a humanized anti-ErbB2 MAb, is active and was recently approved in combination with paclitaxel for the therapy of patients with metastatic ErbB2-overexpressing breast cancer; IMC- C225, a chimeric anti-EGFr MAb, has *shown impressive activity when combined with radiation therapy and reverses resistance to chemotherapy. In addition to antibodies, compounds that directly inhibit receptor tyrosine kinases have shown preclinical activity and early clinical activity has been reported. A series of phase III studies with these antibodies and direct tyrosine kinase inhibitors are ongoing or planned, and will further address the role of these active anti-receptor agents in the treatment of patients with cancer.*

Finally from Calderon and Prins[26]:

Epidermal growth factor (Egf), a secreted peptide, is produced by the luminal epithelial cells in the prostate, and is found at the highest concentration in human prostatic secretions compared to the rest of the body. Epidermal growth factor exerts its effects by binding to its tyrosine kinase receptor, epidermal growth factor receptor (EgfR).

Upon binding, EgfR can homo- or heterodimerize with erbB2 receptors, causing autophosphorylation of its tyrosine residues that in turn activate the phosphatidylinositol 3'-kinase (PI3K), mitogen activated protein kinase (MAPK), or phospholipase C-γ (PLC-γ) signaling cascades. In the developing murine prostate gland, Egf has been shown to mediate its actions through the PLC-γ signaling pathway.

Furthermore, rat UGS explants treated with exogenous Egf showed stimulation of prostate bud

4.2 GENE MUTATIONS

The common gene mutations examined for in thyroid cancer are listed below. We examine some in detail.

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[26] https://www.sciencedirect.com/science/article/pii/B9780128126363000055

Gene	*Function*
GNAS[27] {7}	This locus has a highly complex imprinted expression pattern. It gives rise to maternally, paternally, and biallelically expressed transcripts that are derived from four alternative promoters and 5' exons. Some transcripts contain a differentially methylated region (DMR) at their 5' exons, and this DMR is commonly found in imprinted genes and correlates with transcript expression. An antisense transcript is produced from an overlapping locus on the opposite strand. One of the transcripts produced from this locus, and the antisense transcript, are paternally expressed noncoding RNAs, and may regulate imprinting in this region. In addition, one of the transcripts contains a second overlapping ORF, which encodes a structurally unrelated protein - Alex. Alternative splicing of downstream exons is also observed, which results in different forms of the stimulatory G-protein alpha subunit, a key element of the classical signal transduction pathway linking receptor-ligand interactions with the activation of adenylyl cyclase and a variety of cellular reponses. Multiple transcript variants encoding different isoforms have been found for this gene. Mutations in this gene result in pseudohypoparathyroidism type 1a, pseudohypoparathyroidism type 1b, Albright hereditary osteodystrophy, pseudohypoparathyroidism, McCune-Albright syndrome, progressive osseus heteroplasia, polyostotic fibrous dysplasia of bone, and some pituitary tumors
RET[28](10;11;13;15;16)	This gene encodes a transmembrane receptor and member of the tyrosine protein kinase family of proteins. Binding of ligands such as GDNF (glial cell-line derived neurotrophic factor) and other related proteins to the encoded receptor stimulates receptor dimerization and activation of downstream signaling pathways that play a role in cell differentiation, growth, migration and survival. The encoded receptor is important in development of the nervous system, and the development of organs and tissues derived from the neural crest. This proto-oncogene can undergo oncogenic activation through both cytogenetic rearrangement and activating point mutations. Mutations in this gene are associated with Hirschsprung disease and central hypoventilation syndrome and have been identified in patients with renal agenesis.
AKT1[29] (3)	he serine-threonine protein kinase encoded by the AKT1 gene is catalytically inactive in serum-starved primary and immortalized fibroblasts. AKT1 and the related AKT2 are activated by platelet-derived growth factor. The activation is rapid and specific, and it is abrogated by mutations in the pleckstrin homology domain of AKT1. It was shown that the activation occurs through phosphatidylinositol 3-kinase. In the developing nervous system AKT is a critical mediator of growth factor-induced neuronal survival. Survival factors can suppress apoptosis in a transcription-independent manner by activating the serine/threonine kinase AKT1, which then phosphorylates and inactivates components of the apoptotic machinery. Mutations in this gene have been associated with the Proteus syndrome.

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[27] https://www.ncbi.nlm.nih.gov/gene/2778
[28] https://www.ncbi.nlm.nih.gov/gene/5979
[29] https://www.ncbi.nlm.nih.gov/gene/207

Gene	***Function***
HRAS[30] {2;3)	This gene belongs to the Ras oncogene family, whose members are related to the transforming genes of mammalian sarcoma retroviruses. The products encoded by these genes function in signal transduction pathways. These proteins can bind GTP and GDP, and they have intrinsic GTPase activity. This protein undergoes a continuous cycle of de- and re-palmitoylation, which regulates its rapid exchange between the plasma membrane and the Golgi apparatus. Mutations in this gene cause Costello syndrome, a disease characterized by increased growth at the prenatal stage, growth deficiency at the postnatal stage, predisposition to tumor formation, cognitive disability, skin and musculoskeletal abnormalities, distinctive facial appearance and cardiovascular abnormalities. Defects in this gene are implicated in a variety of cancers, including bladder cancer, follicular thyroid cancer, and oral squamous cell carcinoma.
NRAS[31] {2;3)	This is an N-ras oncogene encoding a membrane protein that shuttles between the Golgi apparatus and the plasma membrane. This shuttling is regulated through palmitoylation and depalmitoylation by the ZDHHC9-GOLGA7 complex. The encoded protein, which has intrinsic GTPase activity, is activated by a guanine nucleotide-exchange factor and inactivated by a GTPase activating protein. Mutations in this gene have been associated with somatic rectal cancer, follicular thyroid cancer, autoimmune lymphoproliferative syndrome, Noonan syndrome, and juvenile myelomonocytic leukemia
BRAF[32] (11;15)	This gene encodes a protein belonging to the RAF family of serine/threonine protein kinases. This protein plays a role in regulating the MAP kinase/ERK signaling pathway, which affects cell division, differentiation, and secretion. Mutations in this gene, most commonly the V600E mutation, are the most frequently identified cancer-causing mutations in melanoma, and have been identified in various other cancers as well, including non-Hodgkin lymphoma, colorectal cancer, thyroid carcinoma, non-small cell lung carcinoma, hairy cell leukemia and adenocarcinoma of lung. Mutations in this gene are also associated with cardiofaciocutaneous, Noonan, and Costello syndromes, which exhibit overlapping phenotypes. A pseudogene of this gene has been identified on the X chromosome.
PIK3CA[33] {2;5;8;10;14;21)	Phosphatidylinositol 3-kinase is composed of an 85 kDa regulatory subunit and a 110 kDa catalytic subunit. The protein encoded by this gene represents the catalytic subunit, which uses ATP to phosphorylate PtdIns, PtdIns4P and PtdIns(4,5)P2. This gene has been found to be oncogenic and has been implicated in cervical cancers.
TP53[34] {2;4;5;6;7;8;10}	This gene encodes a tumor suppressor protein containing transcriptional activation, DNA binding, and oligomerization domains. The encoded protein responds to diverse cellular stresses to regulate expression of target genes, thereby inducing cell cycle arrest, apoptosis, senescence, DNA repair, or changes in metabolism. Mutations in this gene are associated with a variety of human cancers, including hereditary cancers such as Li-Fraumeni syndrome. Alternative splicing of this gene and the use of alternate promoters result in multiple transcript variants and isoforms.

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[30] https://www.ncbi.nlm.nih.gov/gene/3265
[31] https://www.ncbi.nlm.nih.gov/gene/4893
[32] https://www.ncbi.nlm.nih.gov/gene/673
[33] https://www.ncbi.nlm.nih.gov/gene/5290
[34] https://www.ncbi.nlm.nih.gov/gene/7157

Gene	***Function***
KRAS[35] {2;3;4)	This gene, a Kirsten ras oncogene homolog from the mammalian ras gene family, encodes a protein that is a member of the small GTPase superfamily. A single amino acid substitution is responsible for an activating mutation. The transforming protein that results is implicated in various malignancies, including lung adenocarcinoma, mucinous adenoma, ductal carcinoma of the pancreas and colorectal carcinoma. Alternative splicing leads to variants encoding two isoforms that differ in the C-terminal region.
CTNNB1[36] (3)	The protein encoded by this gene is part of a complex of proteins that constitute adherens junctions (AJs). AJs are necessary for the creation and maintenance of epithelial cell layers by regulating cell growth and adhesion between cells. The encoded protein also anchors the actin cytoskeleton and may be responsible for transmitting the contact inhibition signal that causes cells to stop dividing once the epithelial sheet is complete. Finally, this protein binds to the product of the APC gene, which is mutated in adenomatous polyposis of the colon. Mutations in this gene are a cause of colorectal cancer (CRC), pilomatrixoma (PTR), medulloblastoma (MDB), and ovarian cancer
PTEN[37] {1;3;6;7;8)	This gene was identified as a tumor suppressor that is mutated in a large number of cancers at high frequency. The protein encoded by this gene is a phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase. It contains a tensin like domain as well as a catalytic domain similar to that of the dual specificity protein tyrosine phosphatases. Unlike most of the protein tyrosine phosphatases, this protein preferentially dephosphorylates phosphoinositide substrates. It negatively regulates intracellular levels of phosphatidylinositol-3,4,5-trisphosphate in cells and functions as a tumor suppressor by negatively regulating AKT/PKB signaling pathway. The use of a non-canonical (CUG) upstream initiation site produces a longer isoform that initiates translation with a leucine, and is thought to be preferentially associated with the mitochondrial inner membrane. This longer isoform may help regulate energy metabolism in the mitochondria. A pseudogene of this gene is found on chromosome 9. Alternative splicing and the use of multiple translation start codons results in multiple transcript variants encoding different isoforms

4.2.1 BRAF V600

BRAF is a RAF gene (protein) which plays a role in many pathways and has been a key player in many malignancies. Mutations in somatic cells of BRAF have resulted in multiple malignancies. These mutations have been found in many malignancies such as melanoma, prostate and breast.

As noted:

The management and treatment of malignant thyroid micro nodules (i.e., PTMC) can be a challenge for physicians. Most PTMC are indolent and have an excellent prognosis; however, a subgroup shows an aggressive biological and clinical behavior similar to PTC. While additional robust prospective studies are required, there is now a body of evidence suggesting that BRAF V600E-positive PTMCs show aggressive behavior, whereas BRAFV600E-negative PTMCs have a good prognosis.

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[35] https://www.ncbi.nlm.nih.gov/gene/3845
[36] https://www.ncbi.nlm.nih.gov/gene/1499
[37] https://www.ncbi.nlm.nih.gov/gene/5728

This suggests that it will be valuable to consider the BRAF V600E mutation as a prognostic marker of PTMC aggressiveness and to undertake prospective studies with systematic screening for the BRAF V600E mutation and long-term follow-up to validate this marker of tumor aggressiveness.

As Ascierto et al have noted:

BRAF is a serine/threonine protein kinase, encoded on chromosome 7q34, that activates the MAP kinase/ERKsignaling pathway. BRAF is the family member most easily activated by Ras. In addition, the basal kinase activity of BRAF is higher than that of other family members. This provides a potential rationale for the frequent mutational activation of BRAF observed in human tumors. In fact, approximately 50 % of melanomas harbor activating BRAF mutations.

Among the BRAF mutations observed in melanoma, over 90 % are at codon 600, and among these, over 90 % are a single nucleotide mutation resulting in substitution of **glutamic acid for valine** *(BRAFV600E: nucleotide 1799 T>A; codon GTG>GAG).*

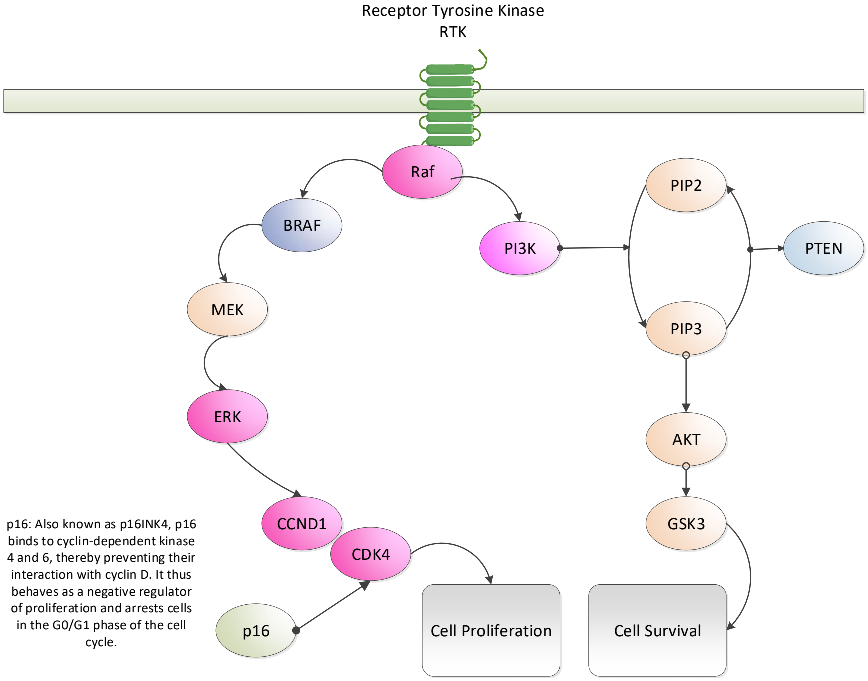
The second most common mutation is BRAFV600K substituting **lysine for valine***, that represents 5-6 % (GTG>AAG),*

followed by BRAFV600R (GTG>AGG),

an infrequent two-nucleotide variation of the predominant mutation, BRAF V600 'E2' (GTG>GAA), and BRAF V600D (GTG>GAT).

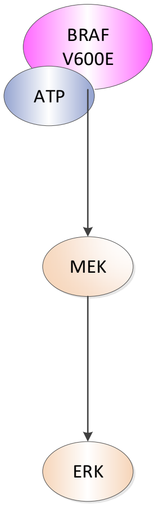
The prevalence of BRAFV600K has been reported as higher in some populations

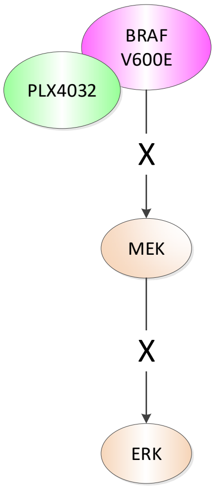
Note in the Figure below the BRAF functions:



p16: Also known as p16INK4, p16 binds to cyclin-dependent kinase 4 and 6, thereby preventing their interaction with cyclin D. It thus behaves as a negative regulator of proliferation and arrests cells in the G0/G1 phase of the cell cycle.

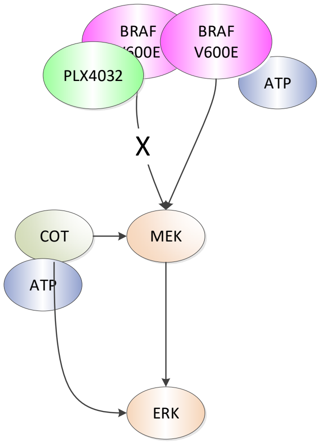
BRAF V600 blocking can be achieve as shown below:





Note: PLX4032 replaces the activating ATP and deactivates the V600E BRAF.

with the following details:



From Solit and Rosen Fig 1: *"the overexpression of RAF1 or the activation of RAS as a result of RAS mutation or upstream activation of a receptor tyrosine kinase promotes:*

(i) the formation of RAF dimers. In cells expressing RAF dimers, binding of RAF inhibitors to one member of the dimer transactivates the other, nonbound member.

(ii) In such cells, PLX4032 does not inhibit MAP kinase signaling, which leads to drug resistance.

(iii) Alternatively, the overexpression of mitogen-activated protein kinase kinase kinase 8 (MAP3K8, or COT) results in RAF-independent activation of MEK and ERK and thus resistance to PLX4032.

(iv) The activation of upstream receptor tyrosine kinases may also cause resistance to PLX4032 by activating RAS, as well as by activating parallel signaling pathways, which results in diminished dependence of the cell on RAF signaling. PDGFRβ denotes platelet-derived growth factor receptor β, and RAS-GTP RAS in its active, GTP-bound state."

4.2.2 RAS

From Mendelsohn et al

Point mutations in RAS genes are among the most common oncogenic abnormalities in all cancers, and DTC is no different. Mutations in the RAS protein lead to constitutive activation through alterations in the binding affinity of the kinase for GTP or through inactivation of its intrinsic GTPase activity. Thus, mutant RAS can signal downstream through both the MAPK and PI3K/Akt pathways without upstream activation derived from ligand-bound RTK. All three RAS genes (H-RAS, K-RAS, and N-RAS) are implicated in thyroid tumor formation from follicular cells, including 20% to 40% of benign follicular adenomas, 40% to 50% of FTC (including 15% to 20% of oxyphilic variants), 10% to 20% of PTC (almost exclusively follicular variants of PTC), and 25% of PDTC.

The presence of a RAS mutation may portend more aggressive disease with worse outcomes, but this has not been extensively examined.19,23 Each of these histologies has also been observed in transgenic mice expressing RAS mutations, although the presence of mutant RAS proteins alone is likely insufficient to cause tumor formation.24,25
PI3K/Akt Pathway

Inactivating germline mutations of the tumor suppressor gene PTEN cause Cowden syndrome, which carries a 50- to 70-fold increased risk for the development of DTC, especially FTC.26,27 Loss of this tumor suppressor function leads to activation of PI3K, Akt, and mTOR, thus contributing to enhanced cell cycle progression, decreased apoptosis, and increased tumor proliferation. However, mutations in individual genes in this pathway are otherwise uncommonly reported as early oncogenic events.

Instead, somatic mutations and/or overexpression of PIK3CA (which encodes the class I p110α catalytic subunit of PI3K), AKT, and PTEN are observed as frequent later events, especially in FTC, PDTC, and ATC.20,28,29 Gene amplification as well as activating point mutations are

observed in 10% to 20% of PDTC and 40% of ATC and can be found in tumors also bearing either BRAF or RAS mutations. AKT activation is also characteristic of the invasive fronts of aggressive DTC and has been reported to trigger increased cellular motility.30
PAX8/PPARγ

A chromosomal translocation, t(2:3) (q13;p25), results in the PAX8/PPARγ mutation, which couples the DNA binding domains of the thyroid transcription factor PAX8 with the entire coding sequence of the nuclear peroxisome proliferator-activated receptor subtype γ1.31 The actual mechanisms by which the encoded fusion protein contributes to thyroid tumorigenesis remain unclear.

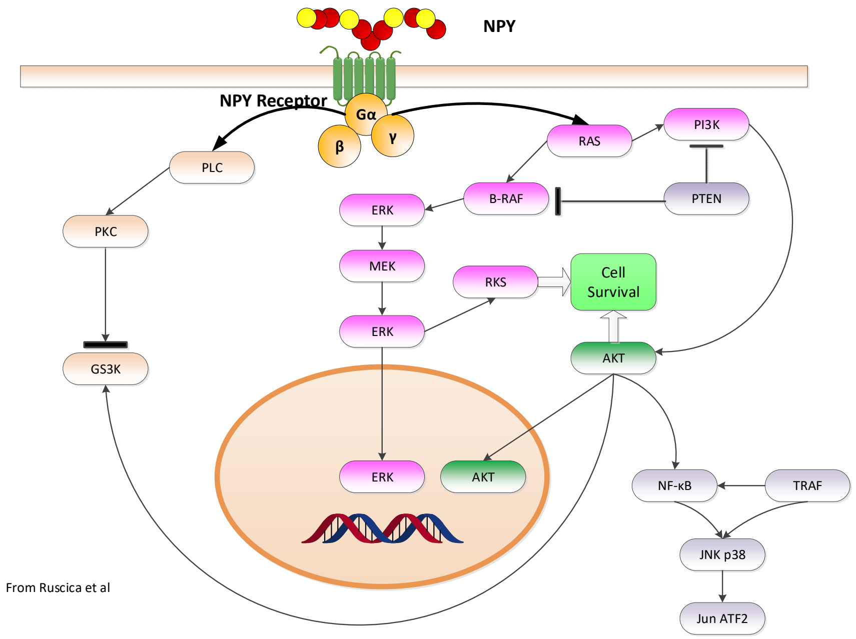
However, several critical pathways may be affected, including reduced expression of PTEN leading to increased activation of Akt, and a dominant-negative effect on the normal PPARγ transcription factor permitting enhanced cellular proliferation and reduction of apoptosis. This mutation may be preferentially seen in younger patients with smaller tumors, which are generally better prognostic signs, but conversely are also seen in tumors with solid or nested histologies as well as with vascular invasion.

From Ruscica et al we have:

The neuropeptide Y (NPY) family of peptides, in addition to its many physiological actions, has also been involved in the modulation of tumor progression, with specific reference to endocrine-related cancers such as neuroendocrine tumors, breast and prostate cancers. These have been found either to express NPY receptors, or to secrete NPY-related peptides, or both.

The study of the role of the NPY family of peptides in the biology of endocrinerelated tumors, specifically concerning cell proliferation, angiogenesis, invasion and metastatization, may help to clarify some aspects of tumor pathophysiology, as well as to indicate novel diagnostic markers and therapeutical approaches…..

Proposed mechanisms of ERK1/2 activation by NPY in human prostate cancer cells. In PC3 cells, NPY activates ERK1/2 via PKC and, possibly, via RAS/RAF, whereas in DU145 cells, PKC activation is not required for NPY-induced ERK1/2 phosphorylation



From Ruscica et al

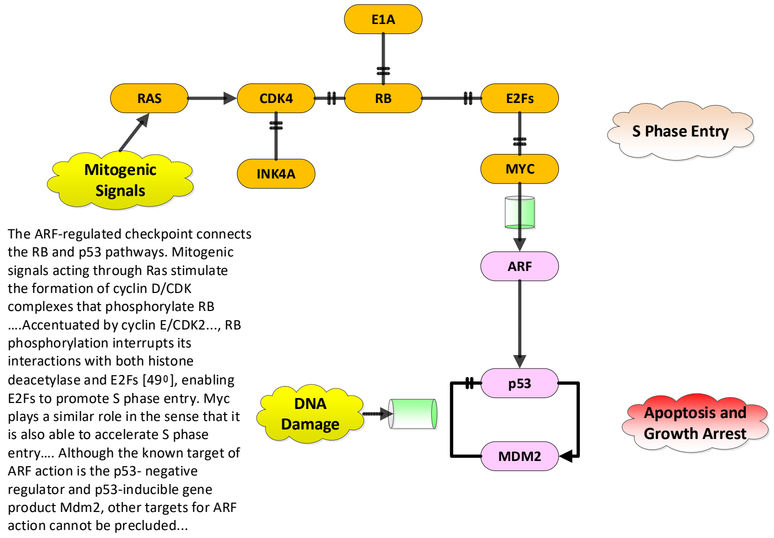
Sherr and Weber discuss the function of RAS as below. The first state regarding the Figure:

The ARF-regulated checkpoint connects the RB and p53 pathways. Mitogenic signals acting through Ras stimulate the formation of cyclin D/CDK complexes that phosphorylate RB in mid to late G1 phase. Accentuated by cyclin E/CDK2 (not shown), RB phosphorylation interrupts its interactions with both histone deacetylase and E2Fs, enabling E2Fs to promote S phase entry. Myc plays a similar role in the sense that it is also able to accelerate S phase entry.

By dampening cyclin D-dependent kinase activity, p16INK4a acts as a potent tumor suppressor. One of the oncogenic effects of adenovirus E1A is to interfere with RB function. Inappropriately increased E2F or Myc signals, stemming from oncogene activation, trigger ARF expression (the vertical barrel designates the checkpoint) and activate p53 to induce either cell-cycle arrest or apoptosis, depending on the biologic context. Although the known target of ARF action is the p53- negative regulator and p53-inducible gene product Mdm2, other targets for ARF action cannot be precluded.

Similarly, Mdm2 probably interacts with proteins other than p53. DNA damage (checkpoints collectively indicated by the horizontal barrel) is shown to access the Mdm2–p53 machinery independently of ARF. However, ARF loss enables Mdm2 to work more efficiently in countering p53 function in response to DNA damage. All proteins illustrated can act as oncoproteins (light shading, black letters) or tumor suppressors (

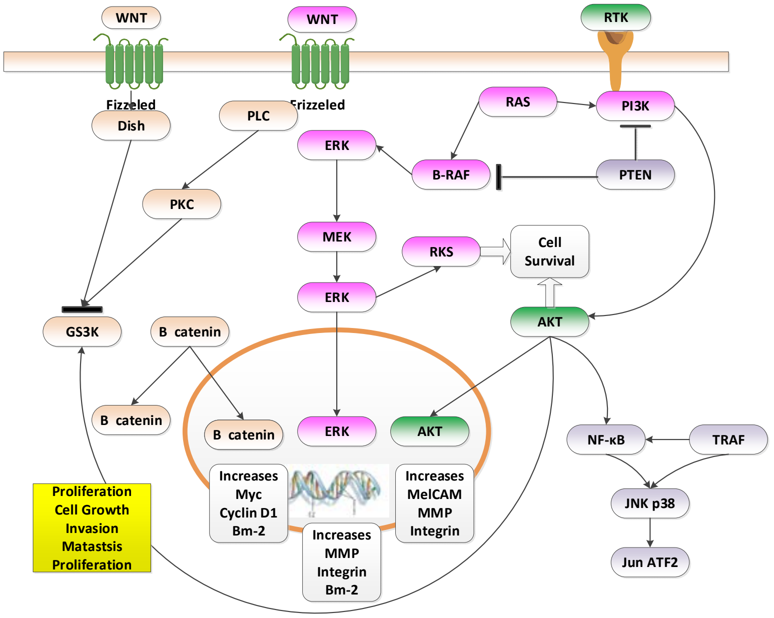
From Sherr and Weber



The ARF-regulated checkpoint connects the RB and p53 pathways. Mitogenic signals acting through Ras stimulate the formation of cyclin D/CDK complexes that phosphorylate RB ….Accentuated by cyclin E/CDK2…, RB phosphorylation interrupts its interactions with both histone deacetylase and E2Fs [49^{0}], enabling E2Fs to promote S phase entry. Myc plays a similar role in the sense that it is also able to accelerate S phase entry…. Although the known target of ARF action is the p53- negative regulator and p53-inducible gene product Mdm2, other targets for ARF action cannot be precluded...

4.2.3 PTEN

PTEN is a major controlling gene. We show the overall pathway elements below.



PTEN is a significant gene which controls the Akt pathway which in turn controls the replication of cells. Loss of PTEN is often seen in metastatic prostate cancer. In many ways it is the hallmark of this change. As stated in NCBI[38]:

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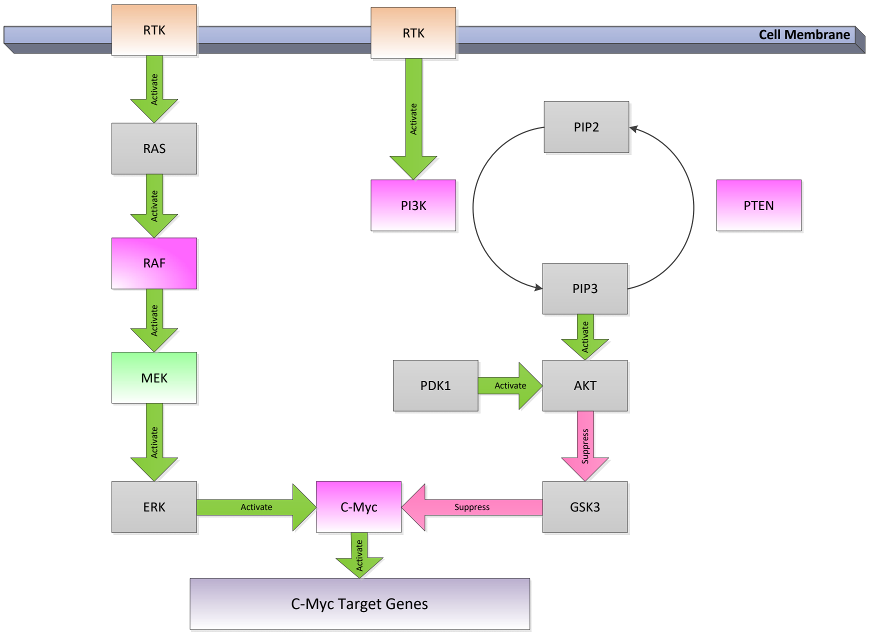
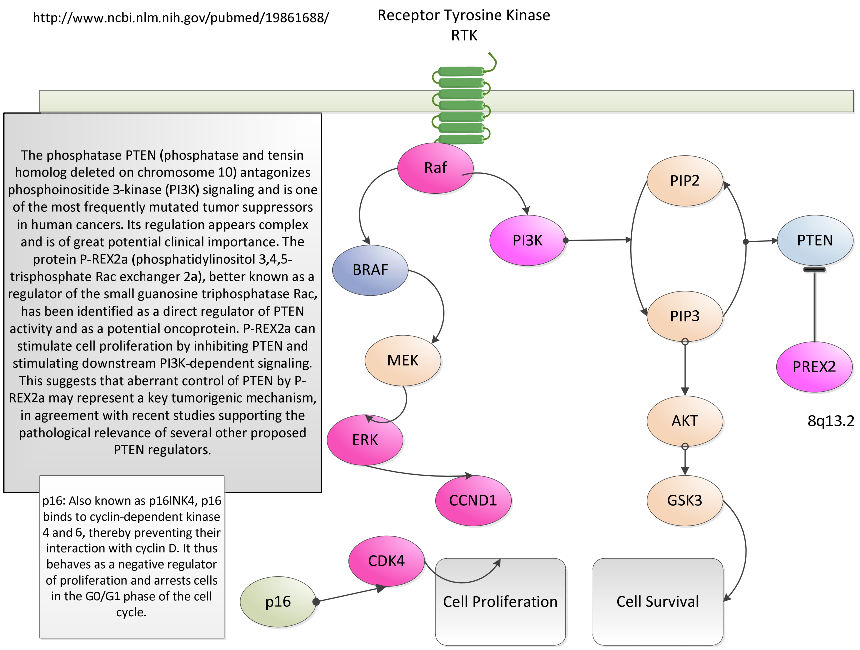
[38] http://www.ncbi.nlm.nih.gov/gene/5728

This gene was identified as a tumor suppressor that is mutated in a large number of cancers at high frequency. The protein encoded this gene is a phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase. It contains a tensin like domain as well as a catalytic domain similar to that of the dual specificity protein tyrosine phosphatases. Unlike most of the protein tyrosine phosphatases, this protein preferentially dephosphorylates phosphoinositide substrates. It negatively regulates intracellular levels of phosphatidylinositol-3,4,5-trisphosphate in cells and functions as a tumor suppressor by negatively regulating AKT/PKB signaling pathway.

First the PTEN pathway as shown below:



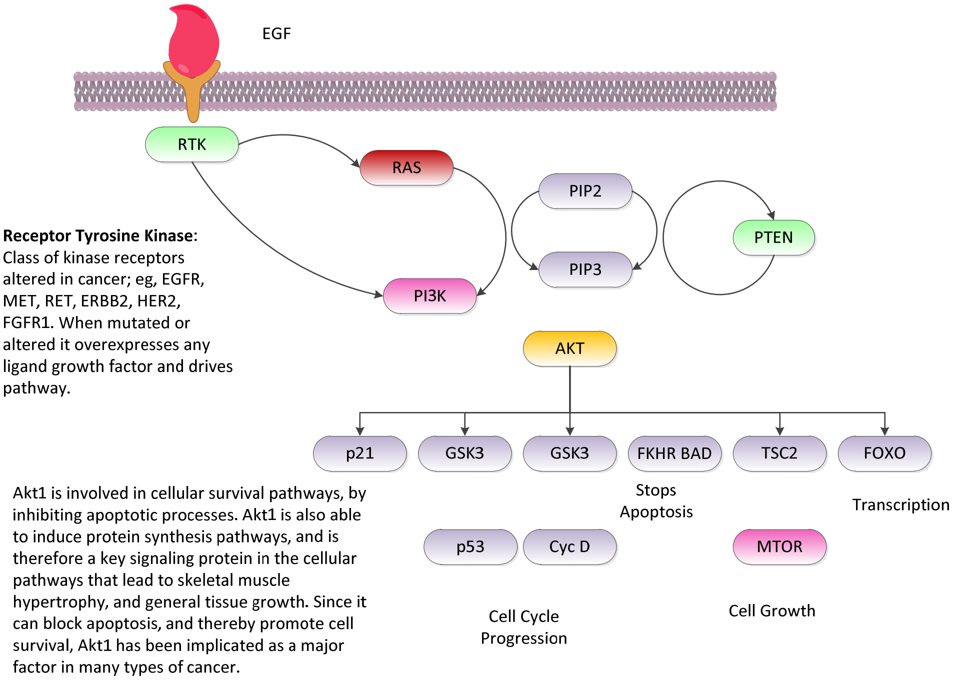
Note PTEN modulates the production of Akt which in turn modulates c-Myc which in turn controls cell reproduction. Any effect which causes PTEN to not be expressed will in turn result in unfettered cell growth.



The phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome 10) antagonizes phosphoinositide 3-kinase (PI3K) signaling and is one of the most frequently mutated tumor suppressors in human cancers. Its regulation appears complex and is of great potential clinical importance. The protein P-REX2a (phosphatidylinositol 3,4,5-trisphosphate Rac exchanger 2a), better known as a regulator of the small guanosine triphosphatase Rac, has been identified as a direct regulator of PTEN activity and as a potential oncoprotein. P-REX2a can stimulate cell proliferation by inhibiting PTEN and stimulating downstream PI3K-dependent signaling. This suggests that aberrant control of PTEN by P-REX2a may represent a key tumorigenic mechanism, in agreement with recent studies supporting the pathological relevance of several other proposed PTEN regulators.

p16: Also known as p16INK4, p16 binds to cyclin-dependent kinase 4 and 6, thereby preventing their interaction with cyclin D. It thus behaves as a negative regulator of proliferation and arrests cells in the G0/G1 phase of the cell cycle.

PTEN has become a key gene in the development of prostate cancer. It controls a pathway leading up to c-myc control and once PTEN is lost the PCa can be considered as very aggressive. Its loss results in an activation of Akt and then c-myc causing uncontrolled cell growth. The pathway is shown below:



Receptor Tyrosine Kinase: Class of kinase receptors altered in cancer; eg, EGFR, MET, RET, ERBB2, HER2, FGFR1. When mutated or altered it overexpresses any ligand growth factor and drives pathway.

Akt1 is involved in cellular survival pathways, by inhibiting apoptotic processes. Akt1 is also able to induce protein synthesis pathways, and is therefore a key signaling protein in the cellular pathways that lead to skeletal muscle hypertrophy, and general tissue growth. Since it can block apoptosis, and thereby promote cell survival, Akt1 has been implicated as a major factor in many types of cancer.

As Jelovac and Park state[39]:

The phosphatase and tensin homolog gene (PTEN) is a tumor suppressor located on the human chromosome 10q arm and is an important mediator of carcinogenesis in a variety of human malignancies. By the strictest definition, a tumor suppressor is a gene whose loss confers an increased lifetime risk of developing tumors. The most illustrative examples of genes that fulfill this criterion are those associated with familial cancer syndromes whereby heritable inactivation of 1 allele and subsequent increased tumor risk is passed along to each generation in an autosomal-dominant fashion.

Using this as a framework, PTEN is a bona fide tumor suppressor gene in that heritable germline mutations have been described in Cowden syndrome (CS), giving rise to a number of human tumors and cancers, most notably thyroid and breast cancers. As is the paradigm of tumor suppressor genes, affected patients with CS inherit 1 mutant inactive copy of PTEN from either parent, and the ensuing loss of the second allele results in tumor formation with subsequent genetic events that eventually lead to cancer. Although there are notable exceptions to this model, most heritable cancer syndromes are believed to adhere to this pattern.

From the work of McMenamin et al we have the slides below. Here is a case where PIN is still expressing PTEN but as we increase the grade of PCa we see the elimination of PTEN expression. Thus we can say that PIN is a state prior to PTEN suppression and a corollary may be that PCa aggressiveness is reflective of loss of PTEN and activation of Akt pathway.

4.2.4 p53

p53 is one of the earliest gene products that has been correlated with cancers. First known as an oncogene it was found that its function actually inhibited cancerous growth. As Malaguarnera et al have noted:

At variance with other human malignancies, p53 mutations are not frequent in thyroid cancer and are believed to be responsible mainly for cancer progression to poorly differentiated and aggressive phenotype. p63 and p73, two proteins with a high degree of homology with p53, are overexpressed in thyroid cancer, but their role in cancer initiation or progression is controversial. Regulation of p53 family protein function depends on:

(1) the balance between the expression of transcriptionally active (p53, TAp63, and TAp73) and inactive isoforms (DNp63 and DNp73);

(2) their interaction and competition at DNA-responsive elements;

(3) their interaction with regulatory proteins, either inhibitory or activating.

In thyroid cancer, therefore, although mutations of the p53 oncosuppressor protein family are rare, other mechanisms are present, including aberrant expression of p53 family dominant

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[39] http://jama.ama-assn.org/content/304/24/2744.full

negative isoforms, up-regulation of inhibitory proteins, and functional inhibition of activating proteins. The overall result is a defective oncosuppressor activity.

These inactivating mechanisms may be present in the early stages of thyroid cancer and in different cancer histotypes. A better understanding of this complex network may not only ameliorate our comprehension of cancer biology, but also open the possibility of innovative diagnostic procedures and the development of targeted therapies.

Thus the p53 mutation may not be common its presence can be highly suggestive of a severe malignancy.

4.2.5 GNAS

GNAS appears to be a protein common to hormone glands. From the NLM database:[40]

The GNAS gene provides instructions for making one component, the stimulatory alpha subunit, of a protein complex called a guanine nucleotide-binding protein (G protein).Each G protein is composed of three proteins called the alpha, beta, and gamma subunits. In a process called signal transduction, G proteins trigger a complex network of signaling pathways that ultimately influence many cell functions by regulating the activity of hormones.

The G protein made with the subunit produced from the GNAS gene helps stimulate the activity of an enzyme called adenylate cyclase. This enzyme is involved in controlling the production of several hormones that help regulate the activity of endocrine glands such as the thyroid, pituitary gland, ovaries and testes (gonads),and adrenal glands. Adenylate cyclase is also believed to play a key role in signaling pathways that help regulate the development of bone (osteogenesis). In this way, the enzyme helps prevent the body from producing bone tissue in the wrong place (ectopicbone).

4.2.6 RET

RET appears to be an influential gene involved in thyroid malignancies. From NLM database:[41]

The RET gene provides instructions for producing a protein that is involved in signaling within cells. This protein appears to be essential for the normal development of several kinds of nerve cells, including nerves in the intestine (enteric neurons) and the portion of the nervous system that controls involuntary body functions such as heart rate (the autonomic nervous system). The RET protein is also necessary for normal kidney development and the production of sperm (spermatogenesis).

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[40] https://ghr.nlm.nih.gov/gene/GNAS

[41] https://ghr.nlm.nih.gov/gene/RET

The RET protein spans the cell membrane, so that one end of the protein remains inside the cell and the other end projects from the outer surface of the cell. This positioning of the protein allows it to interact with specific factors outside the cell and to receive signals that help the cell respond to its environment.

When molecules that stimulate growth and development (growth factors) attach to the RET protein, a complex cascade of chemical reactions inside the cell is triggered. These reactions instruct the cell to undergo certain changes, such as dividing or maturing to take on specialized functions.

From Mendelsohn we have:

About 20% of MTC occurs in one of several familial syndromes: multiple endocrine neoplasia (MEN) 2A (which also includes parathyroid tumors and pheochromocytomas); MEN 2B (which also includes pheochromocytomas, intestinal ganglioneuromatosis, neuromas of the tongue and subconjunctiva, and Marfanoid habitus); and familial MTC (FMTC, which lacks the other clinical features of MEN 2A).

Additional variants of MEN 2A have been reported that include cutaneous lichen amyloidosis and with Hirschsprung disease. Germline mutations in RET were identified as causative of these hereditary forms of MTC in two landmark 1993 studies.34,35 Today, more than 99% of all cases of hereditary MTC can be attributed to one of numerous point mutations in RET that cause activation of the tyrosine kinase function of the RTK (Table 43-1). Given the ubiquitous nature of the mutation, it is not surprising that the disease begins with diffuse hyperplasia of all of the C cells, with eventual development of one or more malignant foci.

The most common germline mutation, a cysteine-to-arginine substitution at codon 634 (denoted C634R), accounts for at least half of all cases of MEN 2A and has also been extensively studied in vitro in the well-characterized TT cell line.36 This mutation is found in the cysteine-rich extracellular domain of RET, a region responsible for ligand-dependent dimerization. However, in the setting of the C634R mutation, RET is capable of ligand-independent dimerization, leading to autophosphorylation of the intracellular tyrosine residues that are responsible for interaction with downstream signaling pathways.

In contrast, a methionine-to-threonine substitution at codon 918 (denoted M918T) is associated with the more aggressive phenotype of MEN 2B. The M918T mutation occurs in the intracellular domain of RET, changing the conformation of the tyrosine kinase domain and allowing marked enhancement of autophosphorylation in the absence of dimerization. In addition, allelic imbalance, due to either increased copy number of the mutant RET allele or deletion of part or all of the wild-type allele, has been reported in several cases of MEN 2A as well as the TT cell line itself.

Sporadic MTC, on the other hand, is not associated with germline changes in RET, but nonetheless, somatic RET mutations have been commonly reported in 25% to 50% of sporadic MTC cases. In this instance, the most frequent somatic mutation is the M918T alteration, but numerous other codon changes have also been observed, including selected deletions as well as point mutations. Of note, about 6% to 7% of patients with clinically sporadic MTC are found to *carry germline mutations diagnostic of hereditary forms of the disease despite the absence of a positive family history, thus leading to the consensus recommendation to recommend RET germline testing for all newly diagnosed cases of apparently sporadic MTC.37,38*

Extensive genotype:phenotype correlations have been established in the two decades since RET was identified as causing MTC. In addition to identifying specific clinical syndromes associated with each mutation, these analyses have also demonstrated that disease penetrance, typical age of development of C-cell hyperplasia and malignancy, and the aggressiveness of the malignancy vary in a manner that is based to a large degree on the individual mutation.

Thus, the intracellular domain mutations, which tend to be associated with the aggressive MTC characteristic of MEN 2B, are also found to cause aggressive sporadic MTC when they occur as somatic mutations. Patients who present with sporadic MTC associated with a somatic M918T mutation of RET have worse outcomes, including overall survival.39 These genotype:phenotype correlations are also useful in determining the role and outcomes of genetic screening in hereditary disease.

Recently published guidelines from the American Thyroid Association divide known RET germline mutations into four risk categories that guide earliest age for RET testing of potential familial carriers, earliest age for recommended first thyroid ultrasound and serum calcitonin testing to detect early presymptomatic evidence of disease, and role for potentially curative prophylactic thyroidectomy.38 Using this type of approach, most young patients identified by prospective genetic screening as carriers for FMTC or MEN 2A can be cured with prophylactic thyroidectomy, although a small percentage remain with biochemical evidence of residual disease.

Mutations of RAS have recently been recognized as common in sporadic MTC in the absence of documented RET mutations.41,42 A wide range of frequency has been reported, however, between 10% and 80% of all RET–wild-type sporadic cases, using differing techniques for identifying RAS mutations. In the largest study, tumor samples from 108 sporadic disease patients without somatic RET mutations were subjected to RAS sequencing, yielding a frequency of 17% in that setting.42 Of the three potential genotype combinations, patients who were (mutant)RAS (wt)RET were more likely to be disease free after a median follow-up of 5 years than those who were (wt)RAS (wt)RET or (wt)RAS (mutant)RET.

As Subbiah et al note:

The receptor tyrosine kinase RET can be oncogenically activated by gene fusions or point mutations. RET fusions occur in a variety of malignancies, including 1%-2% of lung cancers, up to 10%-20% of papillary thyroid cancers, and rarely in many other solid tumors. RET mutations affect most medullary thyroid cancers (MTCs) , and next generation sequencing (NGS) analysis of large numbers of patient tumors has uncovered RET alterations at low frequency in other tumor types. Such alterations possess the hallmarks of cancer drivers: constitutive kinase and signaling activity, transformation of primary cells, and mutual exclusivity from other drivers. Until recently, only multikinase inhibitors (MKIs) with nonselective RET inhibitory activity have been available for patients with RET-altered cancers. Clinical experience with these nonselective

RET inhibitors has been disappointing, with only modest activity in RET-mutant MTCs and RET fusion-positive lung cancers.

Other MKIs approved for other indications (e.g. sorafenib) possess similar, nonselective anti-RET activity preclinically. In part, this may be due to substantial 'off-target' side-effects that limit the degree of RET-specific inhibition and lead to frequent dose reductions. Together with weak anti- RET potency and poor pharmacokinetic (PK) properties, these limitations prevent potent RET pathway inhibition in patients.

5 MIRNAS

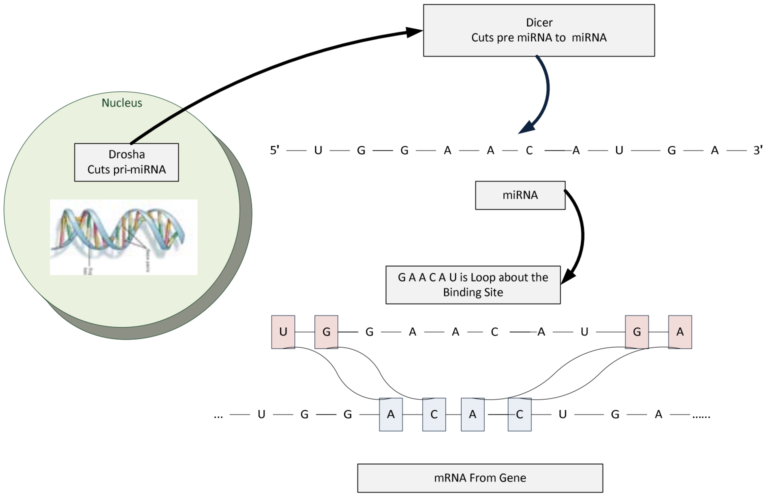
Micro RNAs have been examined for a couple of decades and their function is often to block the translation of mRNA and in turn inhibit certain proteins. We examine them briefly here because we believe that a great deal will be understood about their role in thyroid cancers and in many others.

5.1 BASICS OF MIRNAS

miRNAs are small (19-25 nucleotide single strand RNA) which have been created off intron sections of the DNA of a cell through pol II or pol III. They then operate on mRNA from exons which have escaped from the nucleus and are putatively maturing to proteins in the cytoplasm. Some of the proteins may be beneficial and some may not. The miRNAs seem to be secondary, and in some cases primary, pathway control elements. miRNAs contain RNA nucleotides, U, A, C, G. Thus simply stated if any possible combination is available there could be 4^{22} such miRNAs or about one trillion, equal to the national debt each year! This is a simplistic statement but it does provide a metric. We have discovered just more than a 1,000 miRNAs to data, with variants on some. Therefore a great deal more can be determined[42].

To demonstrate the recent occurrence of miRNA, it was not until the 6th edition of Watson's Biology of the Gene in 2008 that we see a Chapter on controlling RNAs with miRNA (See Chapter 18). In addition even some of the recent literature lends miRNAs a place as a curiosity. In fact the more they are understood the more powerful they become.

In the classic review paper by Esquela-Kerscher, A. and F, Slack, they present an excellent discussion on miRNAs. First we present the overall construct. miRNAs are produced like all RNA and then pass through the Drosha/Pasha complex and emerge from the nucleus as a double RNA with a loop. Dicer cuts the loop creating single strand short RNAs which are the miRNA.



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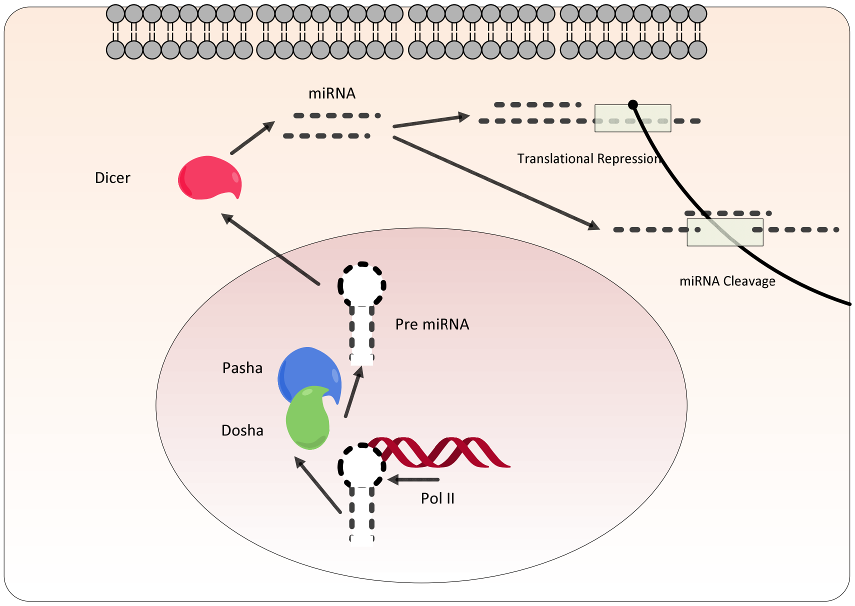
[42] See Giza et al for a summary discussion.

Now from the paper we have the more detailed description where we show how miRNA can interfere with RNA translation by either inhibiting it or by slicing the RNA and in turn also inhibiting it. We depict that below.



We rely upon that here, They state:

The biogenesis of microRNAs. *MicroRNA (miRNA) genes are generally transcribed by RNA Polymerase II (Pol II) in the nucleus to form large pri-miRNA transcripts, which are capped (*$_7$*MGpppG) and polyadenylated (AAAAA). These pri-miRNA transcripts are processed by the RNase III enzyme Drosha and its co-factor, Pasha, to release the ~70-nucleotide pre-miRNA precursor product. (Note that the human let-7a-1 miRNA is shown here as an example of a pre-miRNA hairpin sequence. The mature miRNA sequence is shown in red.)*

RAN–GTP and exportin 5 transport the pre-miRNA into the cytoplasm. Subsequently, another RNase III enzyme, Dicer, processes the pre-miRNA to generate a transient ~22- nucleotide miRNA:miRNA duplex.*

This duplex is then loaded into the miRNA-associated multiprotein RNA-induced silencing complex (miRISC) (light blue), which includes the Argonaute proteins, and the mature single-stranded miRNA (red) is preferentially retained in this complex. The mature miRNA then binds to complementary sites in the mRNA target to negatively regulate gene expression in one of two ways that depend on the degree of complementarity between the miRNA and its target. miRNAs that bind to mRNA targets with imperfect complementarity block target gene expression at the level of protein translation (lower left).

However, recent evidence indicates that miRNAs might also affect mRNA stability (not shown). Complementary sites for miRNAs using this mechanism are generally found in the 32 untranslated regions (3' UTRs) of the target mRNA genes. miRNAs that bind to their mRNA *targets with perfect (or nearly perfect) complementarity induce target-mRNA cleavage (lower right). miRNAs using this mechanism bind to miRNA complementary sites that are generally found in the coding sequence or open reading frame (ORF) of the mRNA target.*

They further detail it as follows:

MicroRNAs can function as tumour suppressors and oncogenes.

a. In normal tissues, proper microRNA (miRNA) transcription, processing and binding to complementary sequences on the target mRNA results in the repression of target-gene expression through a block in protein translation or altered mRNA stability. The overall result is normal rates of cellular growth, proliferation, differentiation and cell death.

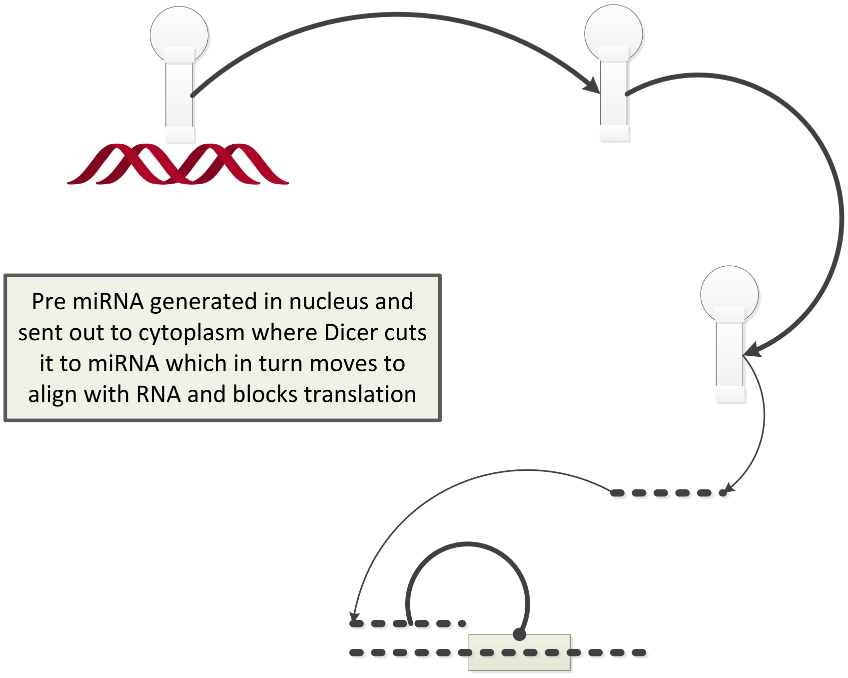
b. The reduction or deletion of a miRNA that functions as a tumour suppressor leads to tumour formation.

A reduction in or elimination of mature miRNA levels can occur because of defects at any stage of miRNA biogenesis (indicated by question marks) and ultimately leads to the inappropriate expression of the miRNA-target oncoprotein (purple squares). The overall outcome might involve increased proliferation, invasiveness or angiogenesis, decreased levels of apoptosis, or undifferentiated or de-differentiated tissue, ultimately leading to tumour formation.

The amplification or overexpression of a miRNA that has an oncogenic role would also result in tumour formation. In this situation, increased amounts of a miRNA, which might be produced at inappropriate times or in the wrong tissues, would eliminate the expression of a miRNA-target tumour-suppressor gene (pink) and lead to cancer progression.

Increased levels of mature miRNA might occur because of amplification of the miRNA gene, a constitutively active promoter, increased efficiency in miRNA processing or increased stability of the miRNA (indicated by question marks). ORF, open reading frame.

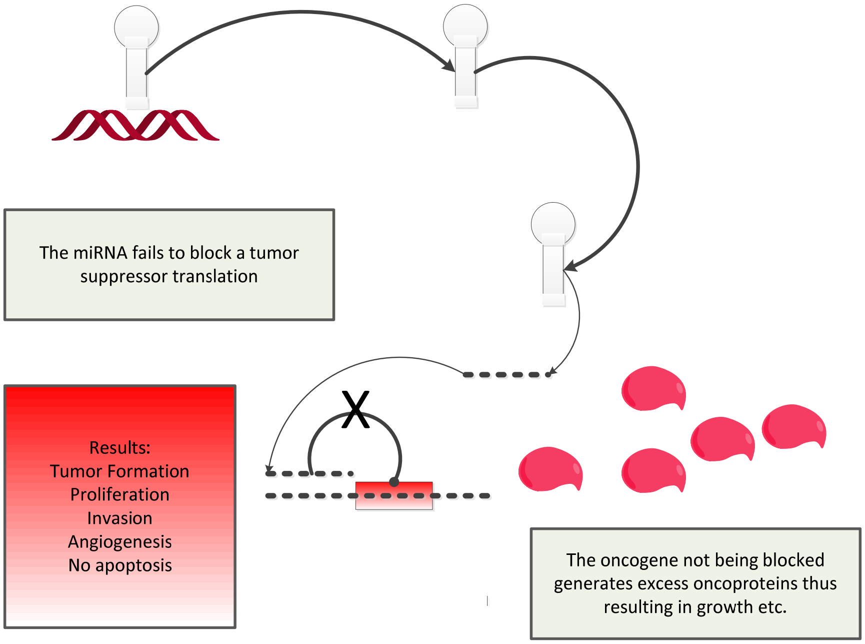
We depict these three cases shown as follows. First, miRNA acting in a normal manner. This is below:



Pre miRNA generated in nucleus and sent out to cytoplasm where Dicer cuts it to miRNA which in turn moves to align with RNA and blocks translation

Notice above the miRNA is assumed to be a normal part of the control mechanism of the control of the conversion of the mRNA into a protein. It block the conversion.

Second, we now consider the second case. Here we have an oncogene which is not blocked by the miRNA and it results in many oncoproteins as shown below.

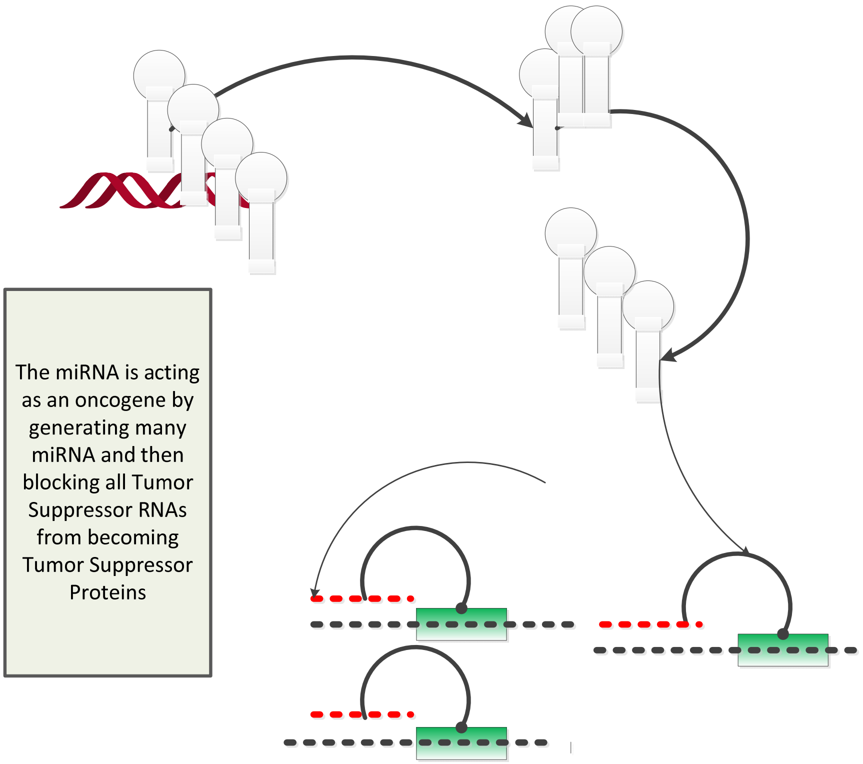


The miRNA fails to block a tumor suppressor translation

Results:
Tumor Formation
Proliferation
Invasion
Angiogenesis
No apoptosis

The oncogene not being blocked generates excess oncoproteins thus resulting in growth etc.

Third and finally in case 2 we have a massive explosion of miRNAs acting as onco activators as shown below.



These methods demonstrate in a somewhat simple manner how the miRNA functions in the case of certain cancers. It also demonstrates how the miRNA can become a target for therapeutics.

5.2 MIRNAS AND EXOSOMES

Exosomes are small vesicles that contain DNA fragments, RNA fragments, miRNA, proteins and other materials ejected from a cell. We give an graphic below. They are small, 10-90 nm in diameter, they are cell like with an shell and material inside, and somehow they can be directed, namely they may have receptors or ligands which can sense what to attach to.

Cells are dynamic entities. The collect stuff from their environment and then expel things back into the environment. Cells may multiply, die off, grow and change. Some of the things cells throw off are shown in the Table below. The exosome is of most interest. It is small, possibly 10 nm or larger .

The exosomes carry various elements around the body and can attach to other cells and transfer their content. Unlike autophagy, they do not digest their contents and unlike many other such things floating about they seem to avoid the attack by the immune system.

These vesicles can convey information, albeit often location independent, and they can also provide communications to other cells, often targeted cells. What do the exosomes resemble?

Are they fully cell like, and do they have ligands and receptors? Receptors may be non-functional unless they do more than just transport their contents. Ligands however would be useful for targeting target cells to deposit their contents. Also how do they manage to deposit the contents when they may attach to a target cell? As Maas et al note:

The topology of EVs is similar to cells, with extracellular receptors and ligands positioned on the outside, and cytoplasmic proteins and RNAs on the inside. Thus, in order for EVs to functionally communicate with cells, different types of interactions may be involved.

This could include release of EV contents in the extracellular space, EV binding to the cell surface, EV-plasma membrane fusion, and uptake by endocytosis. For stimulation of cell signaling by EV-associated extracellular ligands, EVs may directly interact with cognate receptors located on the plasma membrane of cells (or vice versa). This recognition may also serve as a means of "addressing" EVs to certain cell types.

Such ligand-receptor interactions likely accounts for many targeted biological effects of EVs, including those caused by EV-carried growth factors, angiogenic factors and extracellular matrix (ECM) proteins. For delivery of RNAs or cytoplasmic proteins, EVs must not only bind to, but also release their contents into recipient cells, either by direct fusion with the plasma membrane or with the endosomal membrane after endocytosis.

The discussion above regarding the ligand receptors comes up frequently and may be a significant factor when examining the targeting of the exosome. One interesting question may be; does the exosome also take up other materials in its journey? Also, in its intra and extra-vasation, does it act like a neutrophil sensing where to exit, or is it just happenstance? The who issue of exosome interaction is just commencing but will be critical to its understanding.

This is still a complex and poorly defined process but understanding it will be critical for therapeutic uses.

5.3 MIRNAS AND THYROID CANCER

As Xia has noted, the following is a listing of some of the significant miRNAs in thyroid cancers:

miRNA	Genes	Function
miR-138		Loss of expression
miR-21, miR-31, miR-221, miR-222, miR-181b		dys-regulation
miR-30a, miR-125b, miR-26a		Down Regulation
miR-197, miR-346		Follicular thyroid cancer
miR-221, miR-222	p27Kip	Regulate cell cycle

The following miRNAs putatively regulate CSCs.

miRNA	Function
miR-290	Controls *de novo* DNA methylation through regulation of transcriptional repressors in mESs
miR-21	As a REST-regulated miRNA, suppresses the self-renewal of mESs, corresponding to the decreased expression of Oct4,Nanog, Sox2, and c-Myc
miR-24	TGF-regulated miR-24 promotes skeletal muscle differentiation
miR-203	Targets p63 to promote epidermal differentiation by restricting cell proliferation and inducing cell cycle
miR-155	Sustained expression in HSCs causes a myeloproliferative disorder [213]let-7 Regulates BT-IC stem cell-like properties by silencing more than one target
miR-125b	Inhibits osteoblastic differentiation by down-regulation of cell proliferation
miR-26a	Modulates osteogenic differentiation of human adipose tissue-derived stem cells by targeting SMAD1
miR-129	miR-520h miR-520h targets ABCG2 and miR-129 targets EIF2C3 and CAMTA1 important in the development of HSCs
miR-134	Modulates the differentiation of mESs and causes post-transcriptional attenuation of Nanog and LRH1
miR-130a, miR-206	Regulate synthesis of the neurotransmitter substance P in human mesenchymal stem cell-derived neuronal cells
miR-17-92	cluster Promotes cell proliferation and inhibits differentiation of lung epithelial progenitor cells miR-17-5p targets Rbl2
miR-1-2	Regulates cardiac morphogenesis, electrical conduction, and cell cycle
miR-124	Targets laminin and integrin related to neuronal differentiation
miR-1, miR-133, miR-206	Regulate the expression of the alternative splicing factor nPTB during muscle development
miR-181	Targets the homeobox protein Hox-A11 during mammalian myoblast differentiation
miR-9, miR-124a	Modulate embryonic stem cell-derived neurogenesis

Now as Pardini and Calin have noted:

Hormones are messengers circulating in the body that interact with specific receptors on the cell membrane or inside the cells and regulate, at a distal site, the activities of specific target organs. The definition of hormone has evolved in the last years. Hormones are considered in the context of cell–cell communication and mechanisms of cellular signaling.

The best-known mechanisms of this kind are chemical receptor-mediated events, the cell–cell direct interactions through synapses, and, more recently, the extracellular vesicle (EV) transfer between cells. Recently, it has been extensively demonstrated that EVs are used as a way of communication between cells and that they are transporters of specific messenger signals including non-coding RNAs (ncRNAs) such as microRNAs (miRNAs) and long non-coding RNAs *(lncRNAs). Circulating ncRNAs in body fluids and extracellular fluid compartments may have endocrine hormone-like effects because they can act at a distance from secreting cells with widespread consequences within the recipient cells. Here, we discuss and report examples of the potential role of miRNAs and lncRNAs as mediator for intercellular communication with a hormone-like mechanism in cancer …*

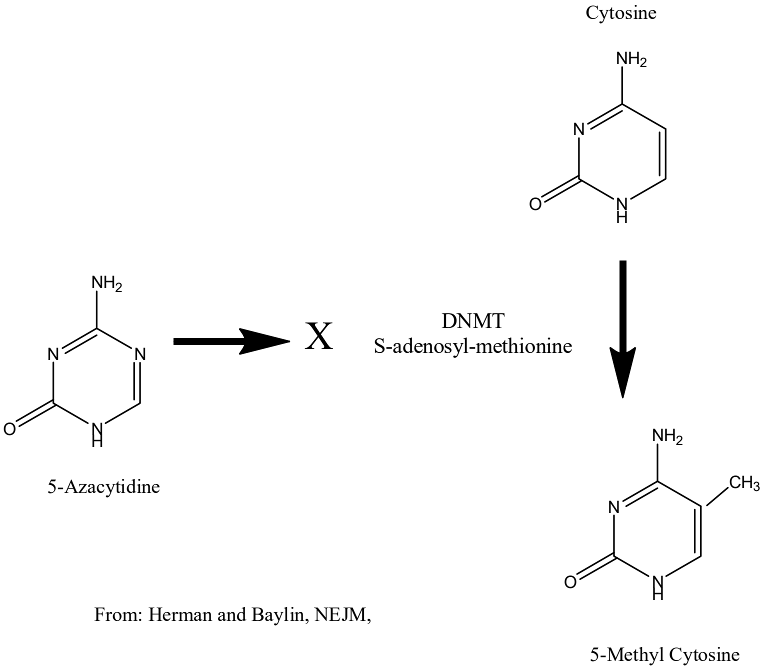
It has been demonstrated that miRNAs can regulate directly genes encoding hormones or other enzymes involved in hormone maturation and metabolism. miRNAs can also target hormone antagonists or receptors indirectly modifying the hormone-mediated cell signaling transmission or could be regulated by hormones either at the level of miRNA transcription and processing. For instance, miR-21 and miR-181-b1 genes are expressed after STAT3 induction, which is activated by interleukin 6 (IL-6). Moreover, miR-21 is repressed by thyroid hormone (TH) and this downregulation regulates GRHL3, a transcriptional inhibitor of type 3 iodothyronine deiodinase (D3) which, in turn, inactivates TH.

6 EPIGENETICS

Epigenetic modifications, other than miRNAs, are often dominated by methylation and acetylation. Methylations can act directly upon the DNA by blocking promoters and even blocking key exons. Methylation and acetylation can change the state of histones and can either open or close sections of DNA to transcription. We examine here what some of these factors may be in the context of thyroid cancers.

6.1 DNA METHYLATIONS

What is methylation? Simply, the attachment of a methyl group to the cytosine molecule creates a methylated C. This is not a complicated process but one which happens frequently and may have significant effects. Cytosine gets methylated and is converted to 5-methyl cytosine. This is accomplished by means of two enzymes as depicted below. This occurs when we have a C and G adjacent. It occurs to the C in that pair. We depict that transition below. Note also that by using 5-Azacytadine we can block that transition.



From: Herman and Baylin, NEJM,

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

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

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

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

As Laird and Jaenisch state:

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

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

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

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

6.1.1 Methylation and Gene Expression

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

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

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

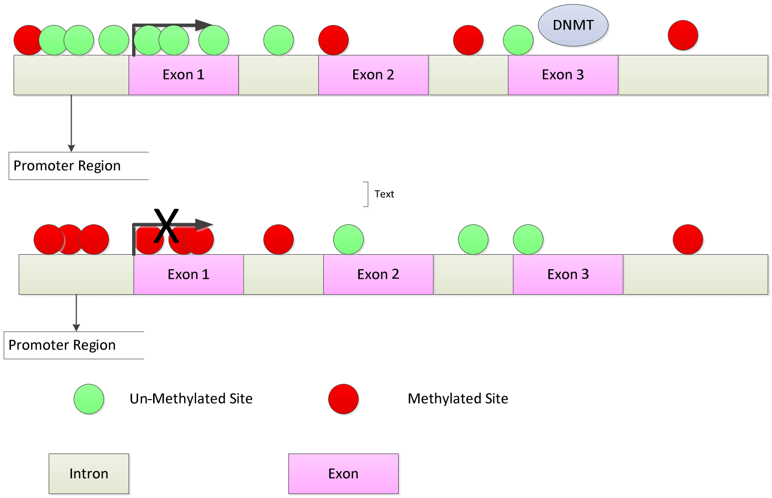
Small regions of DNA, approximately 0.5 to 4.0 kb in size, harbor the expected number of CpG sites and are termed CpG islands. Most of these are associated with promoter regions of *approximately half the genes in the genome (numerous circles surrounding and within exon 1 of the sample gene). In normal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

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

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

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

We depict a modified version of their Figure below:



Thus methylation in this case blocks the expression of the targeted gene.

6.1.2 Methylation and Deamination (C to T)

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

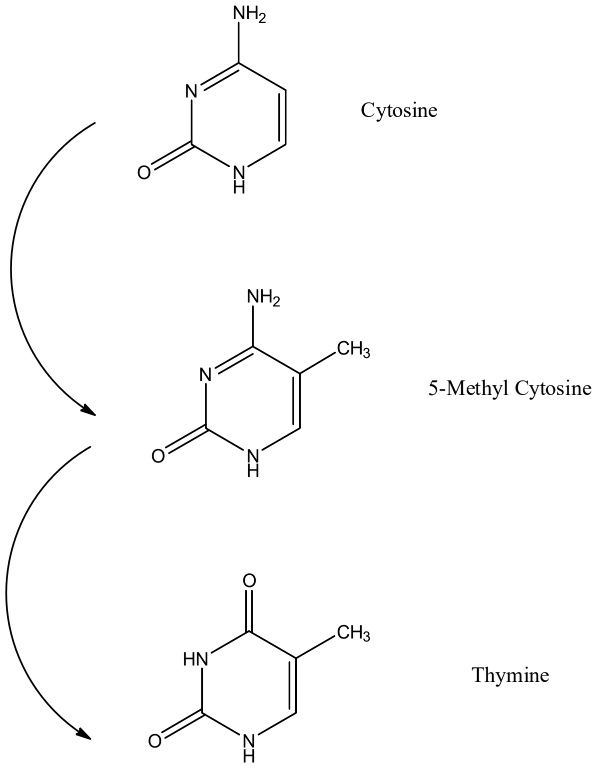
As Herman and Baylin state:

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

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

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

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



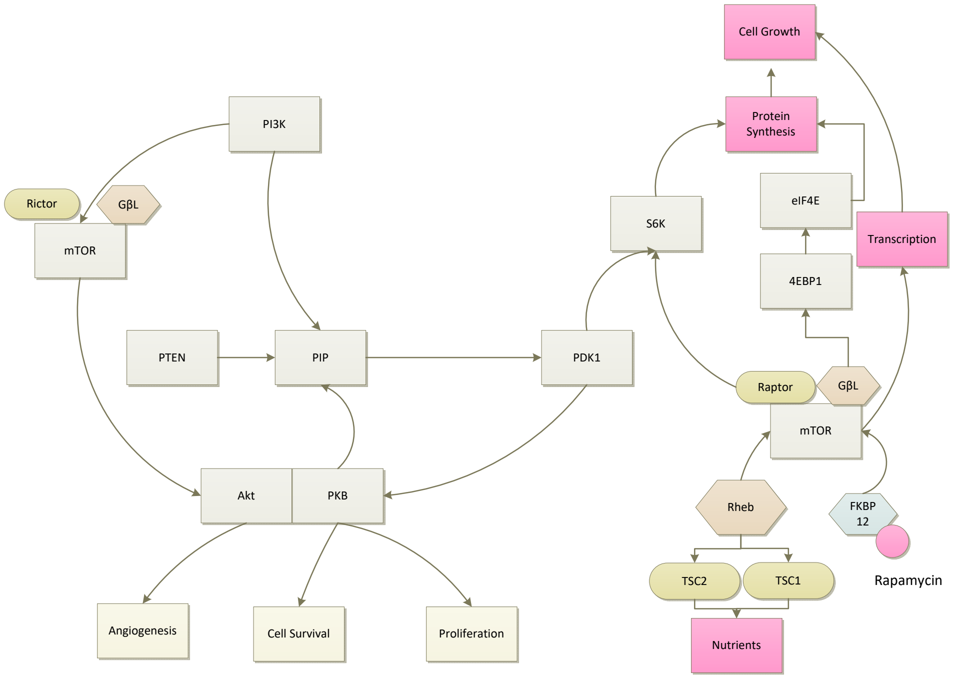
From Robertson (2001) we have some of the genes influenced by methylation or as he states:

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

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

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

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



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

6.1.3 Causes of Methylation

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

As Issa and Kartarjian state:

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

As McCabe et al state:

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

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

McCabe et al continue:

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

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

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

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

Indeed, study of the glutatione peroxidase 1 and 2 double knockout model of inflammatory bowel disease found that 60% of genes that are hypermethylated in colon cancers also exhibit aberrant methylation in the inflamed noncancerous precursor tissues. Although the mechanisms by which DNA damage mediates DNA methylation are not fully understood, O'Hagan and colleagues have examined the process with an engineered cell culture model in which a unique restriction site was incorporated into the CpG island of the E-cadherin promoter.

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

6.2 HISTONE METHYLATION AND/OR ACETYLATION

Histones are the complex protein structures about which the DNA gets wrapped and made inexpressible. When opened the DNA can be read and converted to its ultimate protein wherein action results. Thus histone dynamics and the epigenetics related thereto are critical in many cancers especially the thyroid. Toh et al have discussed this in detail and we demonstrate several cases taken from them herein.

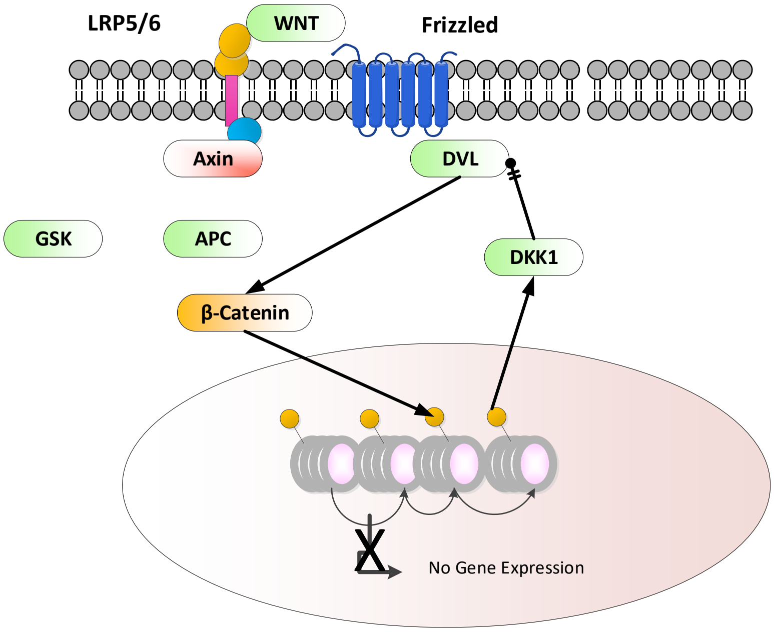
The following three Figures depict histone manipulation from Toh et al. They note related to these:

Regulation of key cancer stem cell signaling pathways by epigenetic mechanisms. Wnt/β-catenin signaling can be enhanced by decreased expression of the DKK1 inhibitor through promoter hypermethylation and increased H3K27me3 and decreased H3K16 acetylation marks. Notch signaling target genes such as Hes1 and Hes5 can be activated by inhibition of H3K27 inhibitory methylation mark at their promoter region by STRAP. Hedgehog signaling pathway can be activated in CSCs epigenetically by Shh promoter hypomethylation and increase HDAC1 expression. Epigenetic deregulation of CSC-related signaling pathways allows cancer cells to acquire self-renewal ability and drug resistance properties

This first we show the WNT signalling and its ability to block DNA expression. The authors note:

The canonical Wnt/β-catenin signaling pathway mediates gene activation through the transcription factor β-catenin. In the absence of Wnt signaling, cytoplasmic β-catenin is inactivated by a degradation complex comprising Adenomatous polyposis coli (APC), Axin, glycogen synthase kinase 3 beta (GSK-3β), and casein kinase 1 (CK1). Phosphorylation by GSK-3β targets β-catenin for ubiquitination and subsequent proteasomal degradation. Upon Wnt ligand binding to Frizzled receptors, the degradation complex is inactivated via low density lipoprotein receptor-related protein 5/6 (LDR5/6) and Dishevelled, allowing for stabilisation of β-catenin. Accumulated β-catenin then translocates into the nucleus, where it associates with T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors to induce transcription of

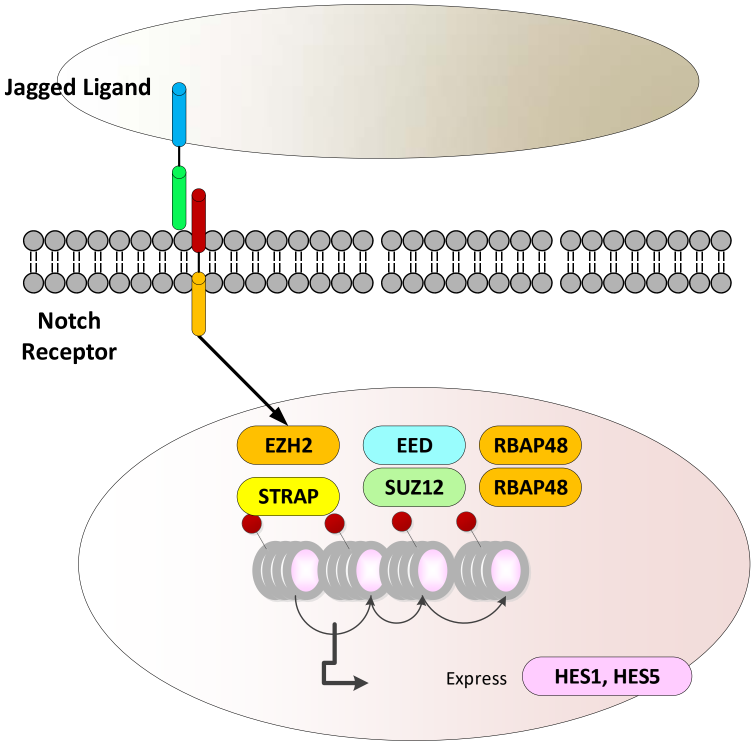
Wnt target genes such as CCND1 and MYC. The Wnt/β-catenin pathway has important functions in normal tissue development and maintenance, as well as in self-renewal and differentiation of CSCs . In fact, the Wnt/β-catenin pathway has been found to be aberrantly activated in a variety of cancers, either via genetic alterations, such as mutations in CTNNB1, APC and AXIN genes, or through epigenetic modulation.



Second we show the Notch signalling in a similar manner.

Notch is a transmembrane receptor involved in cell contact-dependent signaling. Binding of ligands Jagged1/2 or Delta1-4 triggers cleavage of Notch intracellular domain (NICD) by γ-secretase and its release into the cytoplasm. NICD then translocates into the nucleus, where it interacts with recombination signal binding protein for immunoglobulin kappa J region (RBPJ-κ) to transcriptionally induce expression of Notch target genes, such as MYC and HES1 . In the inactive state, RBPJ-κ recruits co-repressor complexes to suppress Notch target genes]. Notch signaling is an evolutionarily conserved pathway that has important roles in development of various tissues and organs.

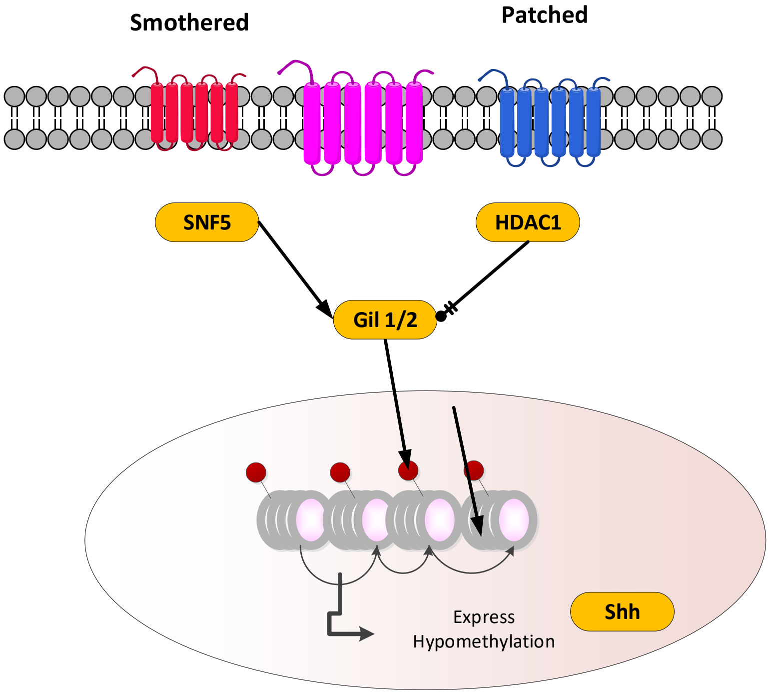
It also regulates cell proliferation and differentiation across a wide range of cell types and during different stages of cell lineage progression. Furthermore, Notch pathway modulates stem cell differentiation and self-renewal. Importantly, Notch signaling has been shown to be crucial for survival of neural stem cells (NSCs). In murine intestinal stem cells, loss of B-lymphoma Mo-MLV insertion region 1 homolog (Bmi1), a target of Notch signaling, decreases proliferation and induces cellular differentiation into goblet cells. Deregulation of Notch pathway has been implicated in various tumors such as prostate cancer, breast cancer, lung cancer, colorectal cancer and haematological malignancies. Recent studies have also reported the role of Notch signaling in breast, colon and oesophageal CSCs.



Finally we show the Hedgehog path for which they note:

The Hedgehog (Hh) signaling pathway plays important roles in guiding cell fate during embryonic development and in maintaining adult tissue homeostasis. It also functions in regulating stem and progenitor cell proliferation and maintenance in several tissues. In the absence of sonic hedgehog ligand (Shh), the Patched receptor (PTCH1) prevents activation of Smoothened (SMO), allowing Gli proteins to be sequestered by suppressor of fused homolog (SUFU) and kinesin family member 7 (Kif7).

Upon Shh binding to PTCH1, SMO is activated and mediates Hh signaling transduction via release of Gli proteins, which then enter the nucleus and act as transcription factors. Gli1 activates transcription of Hh target genes, Gli2 can both activate and repress transcription, while Gli3 functions as a transcriptional repressor.


Smothered
Patched
SNF5
HDAC1
Gil 1/2
Express
Hypomethylation
Shh

7 EMT

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

7.1 BASIC CONCEPTS

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

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

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

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

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

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

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

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

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

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

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

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

As Kong et al have noted:

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

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

7.2 EMT TRANSCRIPTION FACTORS

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

As Mladinich et al note:

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

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

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

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

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

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

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

7.3 SOME SPECIFIC TF

We now consider several specific TF related to EMT functioning.

7.3.1 SLUG

We begin with SLUG.

SLUG[43] (SNAI2, WS2D; SLUGH; SLUGH1; SNAIL2): This gene encodes a member of the Snail family of C2H2-type zinc finger transcription factors. The encoded protein acts as a transcriptional repressor that binds to E-box motifs and is also likely to repress E-cadherin transcription in breast carcinoma. This protein is involved in epithelial-mesenchymal transitions and has antiapoptotic activity. Mutations in this gene may be associated with sporadic cases of neural tube defects.

From Zhao et al:

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

7.3.2 SNAIL

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

SNAIL[44] (SNAI1, SNA; SNAH; SNAIL; SLUGH2; SNAIL1; dJ710H13.1) : The Drosophila embryonic protein snail is a zinc finger transcriptional repressor which downregulates the expression of ectodermal genes within the mesoderm. The nuclear protein encoded by this gene is structurally similar to the Drosophila snail protein, and is also thought to be critical for mesoderm formation in the developing embryo. At least two variants of a similar processed pseudogene have been found on chromosome 2.

As Jagle et al note:

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

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

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

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

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

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

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

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

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

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

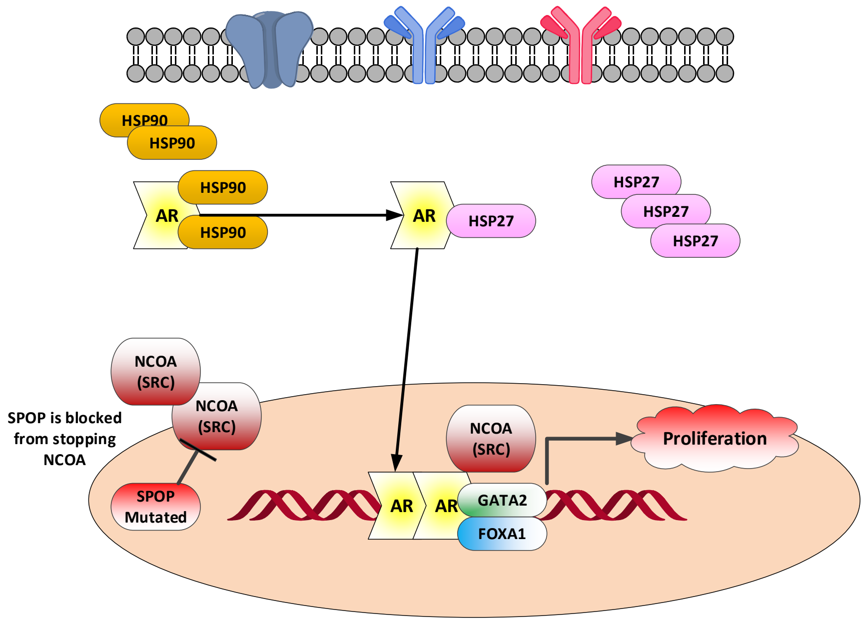
Forkhead box protein A1 (FOXA1) modulates the transactivation of steroid hormone receptors and thus may influence tumor growth and hormone responsiveness in prostate cancer. We therefore investigated the correlation of FOXA1 expression with clinical parameters, prostate-specific antigen (PSA) relapse-free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

However, FOXA1 was overexpressed in metastases and particularly in castration-resistant cases, but was expressed at lower levels in both normal and neoplastic transitional zone tissues. FOXA1 levels correlated with higher pT stages and Gleason scores, as well as with androgen (AR) and estrogen receptor expression. Moreover, FOXA1 overexpression was associated with *faster biochemical disease progression, which was pronounced in patients with low AR levels. Finally, siRNA-based knockdown of FOXA1 induced decreased cell proliferation and migration.*

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

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

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



Now Wyatt and Gleave specifically note regarding FOXA1:

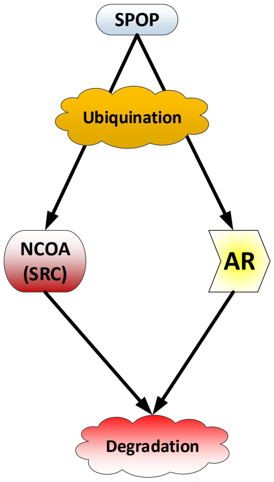
The forkhead protein FOXA1 is a critical interacting partner of the AR, functioning as a pioneer factor to modulate chromatin accessibility and facilitate transcription. In prostate cancer, FOXA1 is capable of specifying unique AR binding sites and has an AR-independent function as a metastasis regulator.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

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

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

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

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



7.3.3 TWIST

TWIST (known also as RS; CSO; SCS; ACS3; CRS1; BPES2; BPES3; SWCOS; TWIST; bHLHa38)[45]: This gene encodes a basic helix-loop-helix (bHLH) transcription factor that plays an important role in embryonic development. The encoded protein forms both homodimers and heterodimers that bind to DNA E box sequences and regulate the transcription of genes involved in cranial suture closure during skull development. This protein may also regulate neural tube closure, limb development and brown fat metabolism. This gene is hypermethylated and overexpressed in multiple human cancers, and the encoded protein promotes tumor cell invasion and metastasis. Mutations in this gene cause Saethre-Chotzen syndrome in human patients, which is characterized by craniosynostosis, ptosis and hypertelorism.

From Mladinich et al:

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

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

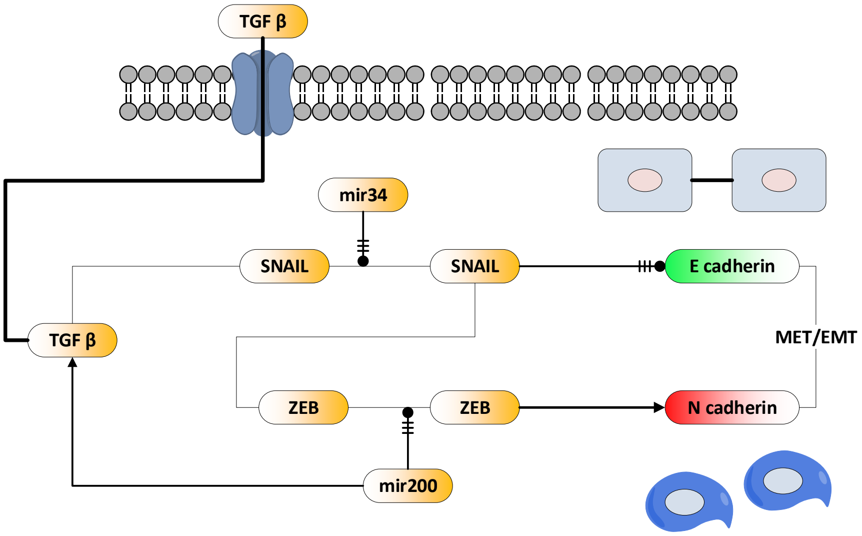
Mechanistically, Twist binds to promoter regions and enhance gene transcription of Slug, subsequently leading to gene repression of E-cadherin Twist can also indirectly repress E-cadherin expression through recruitment of the methyltransferase SET8 that methylates histones for gene silencing.

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

As Yang et al note:

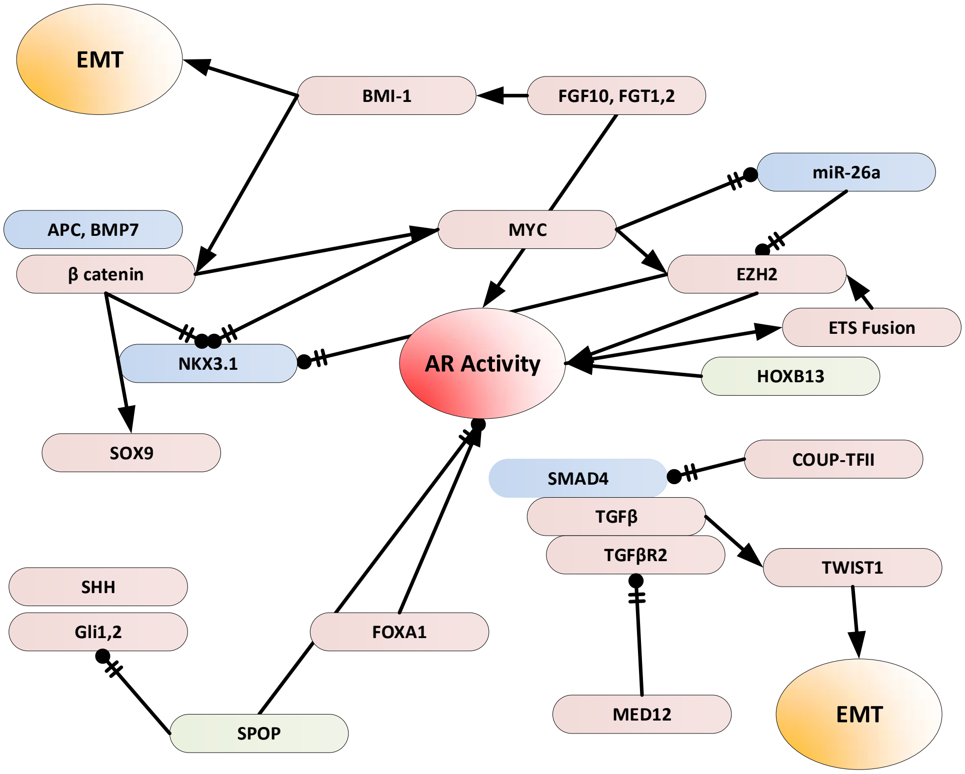
Metastasis is a multistep process during which cancer cells disseminate from the site of primary tumors and establish secondary tumors in distant organs. In a search for key regulators of metastasis in a murine breast tumor model, we have found that the transcription factor Twist, a master regulator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

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

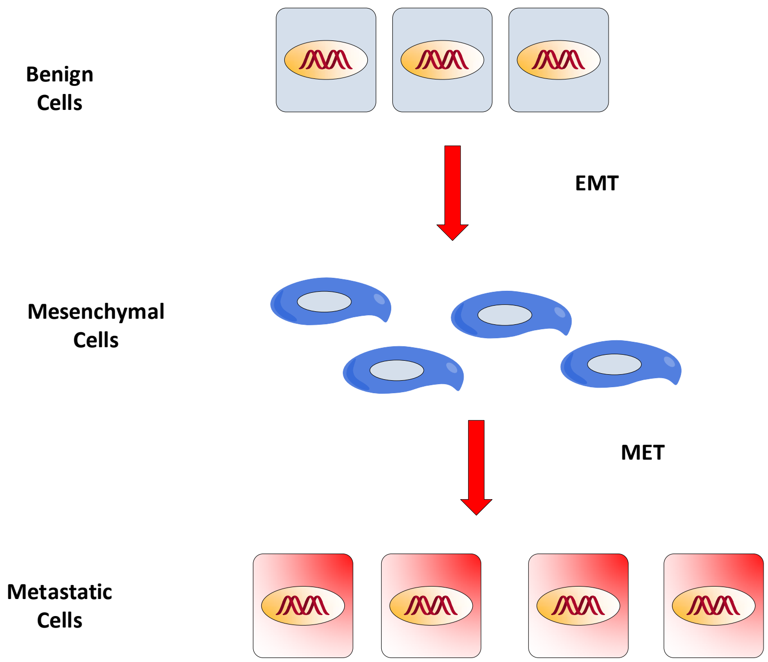


7.4 TOTALITY EMT

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



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


Benign
Cells
EMT
Mesenchymal
Cells
MET
Metastatic
Cells

8 CANCER STEM CELL

There is a somewhat generally accepted understanding that there exists a construct of a cancer stem cell. This seems to be accepted in hematological cancers and has been argued in a multiplicity of solid tumors. For the thyroid there is still a paucity of evidence. Simply a stem cell is one which replicates itself and produces in the process an offspring. The offspring may be a malignant cell but lacks the stem cell ability for self-generation. We examine some of the concepts here as relates to the thyroid carcinomas.

8.1 THE CONCEPT OF A STEM CELL

From Toh et al we have:

Cancer stem cells (CSCs) define a small, unique subset of cells with self-renewal ability and the capacity to generate the different cell types that constitute the whole tumor [1]. These cells are termed CSCs because of their "stem-like" properties commonly shared with normal tissue stem cells. Such properties include extensive selfrenewal ability (symmetrical and asymmetrical) and differentiation capacity. It should be noted that a general capacity to differentiate is not a mandatory feature of CSCs and that the ability of CSCs to differentiate and repopulate the cell types found in the original tumor is of greater significance.

More importantly, CSCs should demonstrate potent tumor-initiation capacity. This property is usually demonstrated by injecting limited number of CSCs into an orthotopic in vivo environment to generate the bulk tumor. Nevertheless, the concept of CSC is of significant importance as it highlights the need to eradicate the CSC populations to achieve an effective cure. The first clear evidence of CSCs being a key tumor initiating subset of cancer cells was demonstrated in acute myeloid leukemia (AML) where prospective CSCs were isolated using cell surface markers that identify normal haematopoietic stem cells and evaluated for their tumor-initiating properties [2, 3]. Since then, similar identifications of tumor-initiating populations have been identified in multiple solid tumors that includes brain, breast, liver, ovary, prostate, lung, melanoma and colon cancers, by using different cell surface markers or through side population (SP) analysis.

As O'Brien et al note:

The cancer stem cell (CSC) or cancer-initiating cancer (C-IC) model has garnered considerable attention over the past several years since Dick and colleagues published a seminal report showing that a hierarchy exists among leukemic cells. In more recent years, a similar hierarchical organization, at the apex of which exists the CSC, has been identified in a variety of solid tumors. Human CSCs are defined by their ability to: (i) generate a xenograft that histologically resembles the parent tumor from which it was derived, (ii) be serially transplanted in a xenograft assay thereby showing the ability to self-renew (regenerate), and (iii) generate daughter cells that possess some proliferative capacity but are unable to initiate or maintain the cancer because they lack intrinsic regenerative potential. The emerging complexity of the CSC *phenotype and function is at times daunting and has led to some confusion in the field. However, at its core, the CSC model is about identifying and characterizing the cancer cells that possess the greatest capacity to regenerate all aspects of the tumor. It is becoming clear that cancer cells evolve as a result of their ability to hijack normal self-renewal pathways, a process that can drive malignant transformation. Studying selfrenewal in the context of cancer and CSC maintenance will lead to a better understanding of the mechanisms driving tumor growth. This review will address some of the main controversies in the CSC field and emphasize the importance of focusing first and foremost on the defining feature of CSCs: dysregulated self-renewal capacity.*

Delerba et al note:

Stem cells are defined by three main properties:

1. differentiation—the ability to give rise to a heterogeneous progeny of cells, which progressively diversify and specialize according to a hierarchical process, constantly replenishing the tissue of short-lived, mature elements;

2. self-renewal—the ability to form new stem cells with identical, intact potential for proliferation, expansion, and differentiation, thus maintaining the stem cell pool;

3. homeostatic control—the ability to modulate and balance differentiation and self-renewal according to environmental stimuli and genetic constraints…..

Three key observations classically define the existence of a CSC population:

1. Only a minority of cancer cells within each tumor are usually endowed with tumorigenic potential when transplanted into immunodeficient mice.

2. Tumorigenic cancer cells are characterized by a distinctive profile of surface markers and can be differentially and reproducibly isolated from nontumorigenic ones by means of flow cytometry or other immunoselection procedures.

3. Tumors grown from tumorigenic cells contain mixed populations of tumorigenic and nontumorigenic cancer cells, thus recreating the full phenotypic heterogeneity of the parent tumor.

8.2 CSC AND EPIGENETICS

Toh et al note:

The canonical Wnt/β-catenin signaling pathway mediates gene activation through the transcription factor β-catenin. In the absence of Wnt signaling, cytoplasmic β-catenin is inactivated by a degradation complex comprising Adenomatous polyposis coli (APC), Axin, glycogen synthase kinase 3 beta (GSK-3β), and casein kinase 1 (CK1). Phosphorylation by GSK-3β targets β-catenin for ubiquitination and subsequent proteasomal degradation.

Upon Wnt ligand binding to Frizzled receptors, the degradation complex is inactivated via low density lipoprotein receptor-related protein 5/6 (LDR5/6) and Dishevelled, allowing for stabilisation of β-catenin. Accumulated β-catenin then translocates into the nucleus, where it associates with T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors to induce transcription of Wnt target genes such as CCND1 and MYC.

The Wnt/β-catenin pathway has important functions in normal tissue development and maintenance, as well as in self-renewal and differentiation of CSCs. In fact, the Wnt/β-catenin pathway has been found to be aberrantly activated in a variety of cancers, either via genetic alterations, such as mutations in CTNNB1, APC and AXIN genes [38–40], or through epigenetic modulation. DNA methylation has been linked to aberrant Wnt/β- catenin pathway activation through the enhanced promoter methylation and subsequent silencing of various Wnt inhibitors such as Wnt inhibitory factor 1 (WIF-1), AXIN2, Secreted frizzled-related protein 1 (SFRP-1), and Dickkopf-related protein 1 (DKK1) in breast and colorectal cancers.

In gastric cancer, Yoda et al. showed that aberrant methylation of Wnt negative regulators, including DKK3, Naked cuticle homolog 1 (NKD1) and SFRP1, could lead to activation of Wnt/β- catenin pathway [44]. Deregulation of Wnt/β-catenin pathway in cancer is also mediated by aberrant histone modifications. Decreased acetylation of H3K16 and increased H3K27 trimethylation along with recruitment of Sirtuin 1 (SirT1), enhancer of zeste homolog 2 (EZH2) and suppressor of zeste 12 protein homolog (Suz12) (components of polycomb repressor complex 2, PCR2) to the promoter of DKK1 inhibited the expression of the DKK1 Wnt antagonist

Cancer Stem Cells ("CSC") and the related cells of origin, and similar cells have been examined in details by many researchers. Simply viewed, a stem cell is a particular cancer cell, whose presence via a plethora of means, it enables the growth and proliferation of the remaining body of a cancer. Removing a cancer stem cell, in principle, removes from the cancer the ability to grow and proliferate. This concept is important for the study of immunotherapy since it reflects an understanding of what cells to target. As relates to immunotherapy, one therefore may target all the other cancer cells but by leaving an active stem cell one allows for a rejuvenation of the malignancy. Our focus here is on prostate cancer since there is somewhat of an intellectual as well as lab based controversy worth examining.

Prostate Cancer, PCa, provides a unique target for examining these cells and at the same time has provided a fertile ground for disputes. Thus there is a strong disagreement as to whether the basal or luminal cell is the cell of origin and therein the CSC issue also arises. In a recent paper from the Tang Lab at MD Anderson they have present conclusions supporting a basal origin. In contrast Shen at Columbia has focused on luminal cells. In this note we attempt to bring an update to what we wrote in 2012 and provide some basis for comparing the various claims[46]. Fundamentally, we take no position in this debate[47].

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[46] See Telmarc White Paper 85, Stem Cells, http://www.telmarc.com/Documents/White%20Papers/85%20Prostate%20Stem%20Cells.pdf and https://www.researchgate.net/publication/301222986_Prostate_Cancer_Stem_Cells?ev=prf_pub

[47] The reader is referenced to the White Papers referenced in this documents for details on specific topics. Also see Prostate Cancer: A Systems Approach by the author.

Zhang et al state:

The prostate gland mainly contains basal and luminal cells constructed as a pseudostratified epithelium. Annotation of prostate epithelial transcriptomes provides a foundation for discoveries that can impact disease understanding and treatment. Here we describe a genome-wide transcriptome analysis of human benign prostatic basal and luminal epithelial populations using deep RNA sequencing. Through molecular and biological characterizations, we show that the differential gene-expression profiles account for their distinct functional properties. Strikingly, basal cells preferentially express gene categories associated with stem cells, neurogenesis and ribosomal RNA (rRNA) biogenesis.

Consistent with this profile, basal cells functionally exhibit intrinsic stem-like and neurogenic properties with enhanced rRNA transcription activity. Of clinical relevance, the basal cell gene-expression profile is enriched in advanced, anaplastic, castration-resistant and metastatic prostate cancers. Therefore, we link the cell-type-specific gene signatures to aggressive subtypes of prostate cancer and identify gene signatures associated with adverse clinical features.

This is an argument for the basal cell being the origin of the CSC. They continue:

The current study has made the following significant findings.

First, our study uncovers unique SC- and EMT-enriched gene-expression profile in unperturbed basal cells that support the long-held hypothesis that the human prostate basal cell layer harbors primitive SCs.

Second, we report the surprising finding that basal cells are enriched in genes normally associated with neurogenesis. In contrast, luminal cells preferentially express proneural genes involved in neural signal response and processing. Consistently, primary basal cells can spontaneously or be induced to undergo 'neural' development in vitro, generating NSC-like cells. Combined with the SC features, these transcriptional programs provide a molecular understanding for the reported basal cell plasticity.

Third, basal cells express high levels of Pol I-associated rRNA biogenesis genes regulated, at least in part, by the MYC transcriptional programme. MYC is often found overexpressed in PCa, especially metastatic PCa. Increased transcription of rRNA genes by Pol I is a common feature of human cancer. Thus, our data may suggest a rationale for treating anaplastic PCa and CRPC with Pol I inhibition, as well as targeting MYC and the MYC-mediated transcriptional programme as a therapy for PCa.

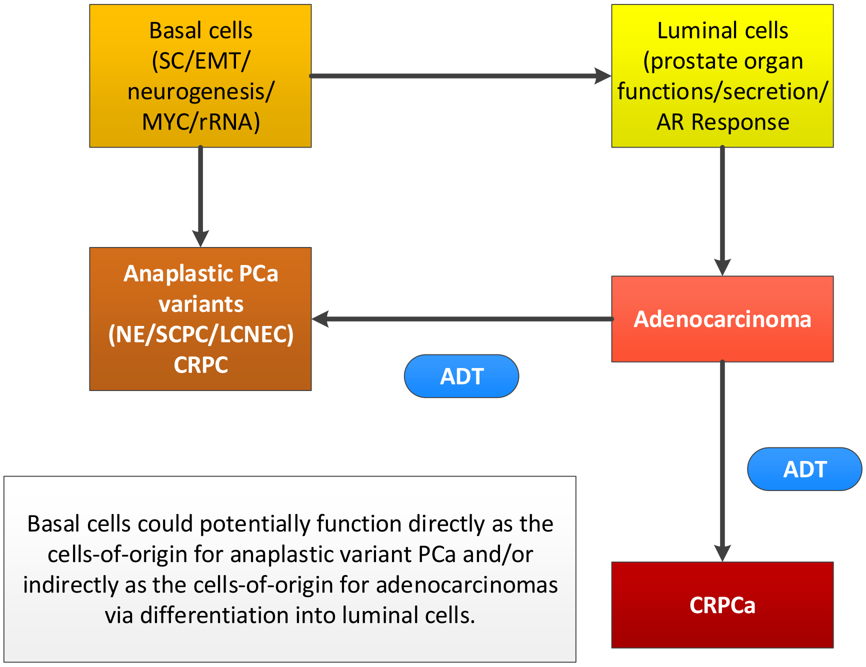
Fourth, our deep RNA-Seq data provide a rich resource for epithelial lineage specific genes and markers in the human prostate.

Fifth, distinct transcriptomes in basal and luminal cells also suggest cross communications between the two epithelial cell types, as well as between the epithelial compartment and the underlying stroma. Understanding such crosstalk will be instrumental for understanding the normal development and tumorigenesis of prostate. Although many of the signaling pathways *mentioned in this study are poorly investigated in normal prostate epithelial biology, their functional involvement in PCa development and progression has been widely documented.*

Last, the basal cell gene-expression profile is linked to adverse clinical features of PCa, indicating a 'biomarker' value of basal cell gene signature for aggressive PCa. Importantly, the molecular resemblance of basal cells to anaplastic PCa and CRPC provides a common molecular understanding of these diverse and poorly characterized aggressive PCa subtypes and implicates basal cells as the cell-of-origin for these variant PCa.

We present the summary of the Tang Lab model. The driver is a basal cell and the luminal cells seem to act if and only if driven by a basal cell process. Furthermore, the neuroendocrine case is shown as a direct and indirect result of the basal driver. We have recently discussed the neuroendocrine prostate cancers in our discussions of pro-NPY[48].

The Figure below is modified from the Wang et al paper and summarizes their concept.



Basal cells could potentially function directly as the cells-of-origin for anaplastic variant PCa and/or indirectly as the cells-of-origin for adenocarcinomas via differentiation into luminal cells.

The above indicates the origin is from basal and then to luminal or the neuroendocrine cells, the latter being substantially less common.

Now the CSC and CCO debate, especially that related to PCa can be viewed in almost classical terms. In the 14th century as Medical Schools at Montpellier, Bologna, Paris and Oxford studied Galen and other then classic medical texts, the use of logic was compelling and demanded. The Trivium, Grammar, Logic and Rhetoric, was required of any student studying the field. This was because studying Galen demanded logic. Processes that were diagnostic or prognostic demanded logical consistency more than phenomenological verification. Strangely in the case of the CSC

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[48] See https://www.researchgate.net/publication/292978295_pro-NPY_PCa_and_Neuroendocrine_Tumors

perhaps logical consistency is pari passu with that phenomenon. One of the major problems is defining the terms in such a manner that they can be consistently phenomenologically compared.

8.3 DEFINITIONS

As we have indicated, one of the more difficult issues when discussing CSCs is the definition. Phenomenological observations have been reduced to definitions and in turn the definitions have been used to search for CSCs. This can be a bit circular at times and may very well be one of the sources of confusion. Let us begin with the paper by Jordan et al from a decade ago in NEJM:

Many studies performed over the past 30 to 40 years, when viewed collectively, have shown that the characteristics of stem-cell systems, the specific stem-cell properties described above, or both, are relevant to some forms of human cancer. Biologically distinct and relatively rare populations of "tumor-initiating" cells have been identified in cancers of the hematopoietic system, brain, and breast.

Cells of this type have the capacity for self-renewal, the potential to develop into any cell in the overall tumor population, and the proliferative ability to drive continued expansion of the population of malignant cells. Accordingly, the properties of tumor-initiating cells closely parallel the three features that define normal stem cells. Malignant cells with these functional properties have been termed "cancer stem cells".

This frankly is a cumbersome definition. They describe stem cells; a necessary part of the definition as follows:

Stem cells occur in many different somatic tissues and are important participants in their physiology. Populations of cells that derive from stem cells are organized in a hierarchical fashion, with the stem cell residing at the apex of the developmental pathway. Stem cells have three distinctive properties: self-renewal (i.e., at cell division, one or both daughter cells retain the same biologic properties as the parent cell), the capability to develop into multiple lineages, and the potential to proliferate extensively. The combination of these three properties makes stem cells unique. The attribute of self-renewal is especially notable, because its subversion is highly relevant to oncogenesis and malignancy. Aberrantly increased self-renewal, in combination with the intrinsic growth potential of stem cells, may account for much of what is considered a malignant phenotype.

Thus we could ask; do all organs have stem cells which are organ specific? We know the skin continually reproduces cells, specifically keratinocytes. Colon cancer has a stem cell element[49].

In the paper by Navin and Hicks they present a taxonomy of possible cancer cell propagation. It is worth examining this before driving towards a de4finition. The facts will ultimately determine the definition. They present the following five categories:

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[49] See Rajasekhar p 274.

1. Clonal: This case is a single cell gets a malignant character and then proceeds with uncontrolled growth. Thus one would expect that each cell in the tumor would reflect this by having genetic homogeneity across any tumor sample. We know that this is clearly not the case.

2. Polyclonal: This is the first case but with a twist. Namely there are several clones clustering together. Thus across any section of the tumor there would be different clones but the clones would be locally consistent. Again this is not the case.

3. Polyclonal via Self-Seeding: This is polyclonal but now the different clones appear across the blood stream as separate but homogeneous entities. Again we know that even as hematopoietic spread occurs there is nonuniform genetic types.

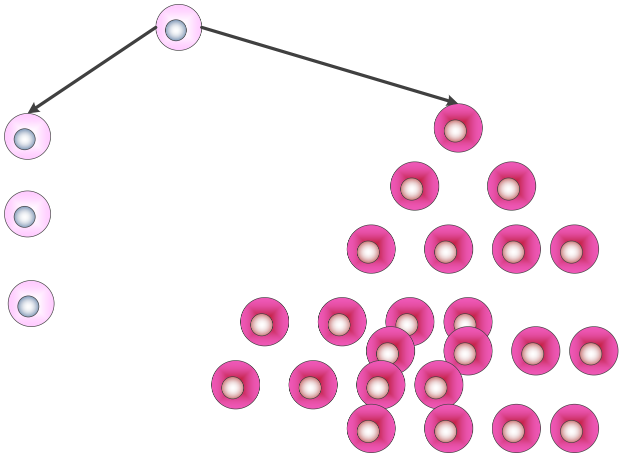
4. Multiple Mutator: This type is total genetic diversity. Here we go from one genotype to another across almost every cell. Thus the genetic diversity is a maximum complexity. Again we know that this is not the case.

5. Cancer Stem Cell: This is the hypothesis of a cancer stem cell. Namely a single genotype both self-replicates and also generates a malignant progeny. The progeny cells are basically the same and they can replicate but do not drive the malignancy. Somehow if one were to remove the CSC the other cells would stop. This may be what happens in certain types of HGPIN as we have speculated based upon HGPIN saturation biopsies resulting in return to full benign state.

Now there is a sixth case not discussed by Navin and Hicks which we have proposed and analyzed using limited data. This is the Markov chain model wherein cells have a genotype but the genotype is subject to random changes at random instants, albeit changes driven by location and complexity of the tumor state. We refer the reader to the results elsewhere.

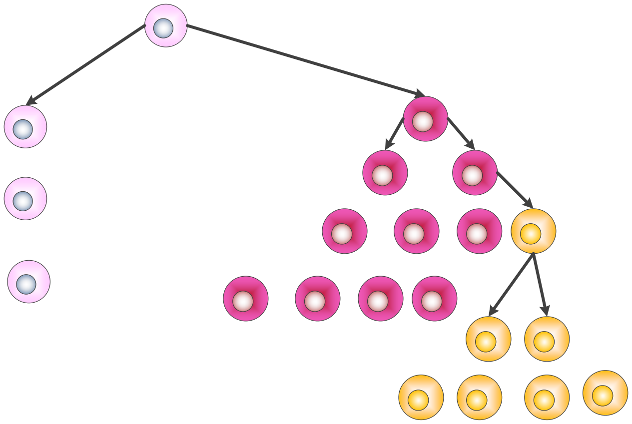
We now demonstrate graphically each of the five cases below:

Case 1: The Clonal Model shows a single mutation and then all subsequent cells are just clones. We have examined this in the case of MDS, Myelodysplastic Syndrome. We know that the malignancy most related to this is AML. We also know that AML appears clonal like but MDS is not the same in all cases. Some have proliferation in red cells while others in platelets. Others may be neutrophil driven. Thus the CSC if there is one is not at the cell base of a core stem cell but at a level above that. Then when AML occurs one may see the CSC moving downward.



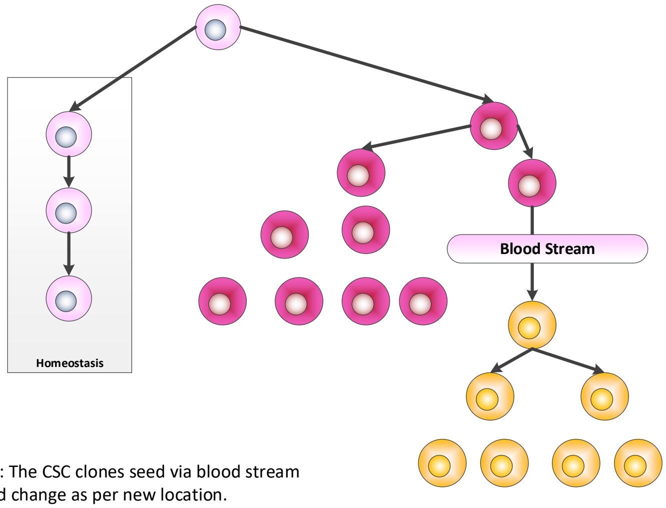
Monoclonal Evolution: Assumes a single malignant clone which continues to replicate

Case 2: Polyclonal. The second model below shows a multiple set of clones. The assumption is that cells continue to mutate but that one an active mutation occurs then the now clone takes off. One would expect to see clusters of common clones.



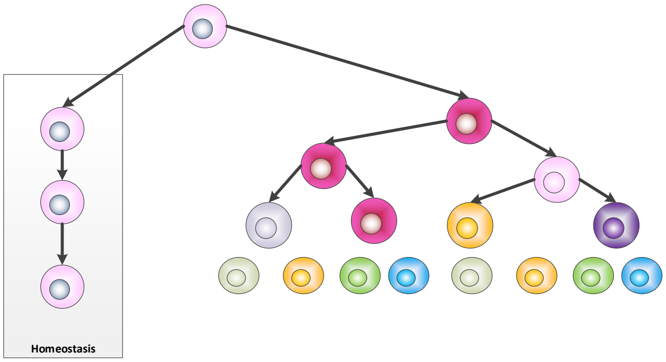
Polyclonal Evolution: Assumes a multiple malignant clones which continues to replicate

Case 3: Self-Seeding. The model below is what they call the self-seeding. This is a polyclonal variant where the clone can change as it moves throughout the body.



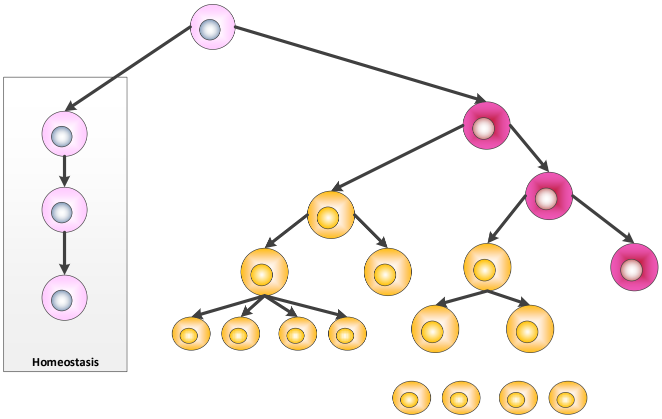
Self Seeding: The CSC clones seed via blood stream and change as per new location.

Case 4: This is the Mutator model wherein cells keep changing is shown below. The end result is a tumor with almost no genomic consistency.



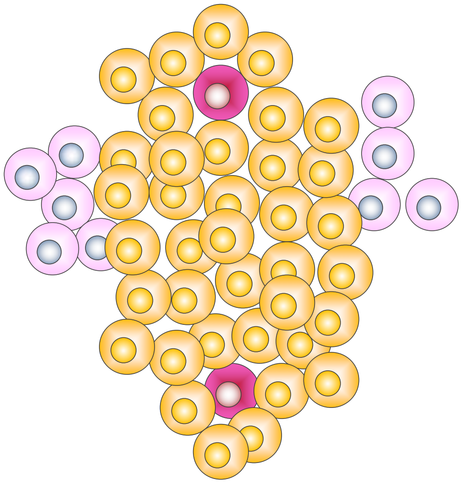
Mutator Phenotype: Generates many variants.

Case 5: This is the case of the CSC. Namely the one cell that starts everything off and keeps it going. We show that below.



Cancer Stem Cell: A single cell self replicates and
also produces malignant but non-driving cells.

Thus if we accept the Navin and Hicks description of the CSC we would expect to see a tumor as shown below. Normal cells at a periphery in a normal homeostatic state, then a large collection of non-stem malignant cells (namely cells which can and do proliferate), and a few CSC which somehow drive the process. One could assume the CSCs drive the proliferation of the non-CSC malignant cells. However, that is open for debate. Furthermore, however, if we were to take the CSC away then in most CSC theories the other malignant cells would undergo apoptosis or some form of cell death.



The previous section used a purely logical descriptive approach for CSC classification. As we noted it did have deficiencies that we have explored elsewhere. However, it does not tell us what a CSC is. There are phenomenological

As it is not experimentally feasible to investigate the potential existence of CSCs in human tumors solely on the basis of these theoretical definitions, CSCs are instead defined in practical terms through the use of several functional assays. The most frequently used methodology involves xenotransplantation of flow sorted populations of primary cancer cells into immunodeficient mice.

In this assay, CSCs are defined as a subpopulation of cells within a primary tumor that can initiate tumor formation in mice following transplantation, unlike the remaining tumor cells. Using this assay, early studies identified CSC populations in hematological malignancies, such as the CD34+CD38- population in acute myeloid leukemia.

Similar approaches were subsequently applied to solid tumors, leading to the identification of candidate CSC populations that were prospectively enriched using specific markers in breast (CD44+CD24-Lin-), brain (CD133+) and colon cancers (CD133+). Overall, however, the available evidence supporting the identification of CSCs in solid tumors has been less convincing, at least in part because solid tumor cells exist in a complex microenvironment that is not readily modeled by xenotransplantation.

Wang and Shen then continue to discuss the issue of definitions:

Much of the confusion in the literature arises through inconsistencies in nomenclature within the field. In particular, due to the wide use of xenotransplantation as a functional assay for CSCs, transformed cells that can initiate tumor formation in this assay are often referred to as CSCs in the literature.

However, a ***tumor initiating cell (TIC) represents a different concept from that of a CSC****, as TICs unquestionably exist within tumors and their identification does not by itself imply a hierarchical organization of a tumor. Indeed, the majority of cells within a tumor could potentially possess TIC properties and nonetheless follow a clonal evolution model.*

Consequently, it is important to distinguish CSCs that have been strictly defined by their position and function within a lineage hierarchy in vivo from CSCs that have been identified as rare TICs in transplantation studies.

A similar confusion arises with respect to the cell of origin for cancer, which corresponds to a normal tissue cell that is the target for the initiating events of tumorigenesis. In principle, a normal adult stem cell could be a logical cell of origin for cancer, as it would retain the ability to self-renew and generate a hierarchy of differentiated lineages within a tumor.

However, it is also possible that a cell of origin could correspond to a downstream progenitor cell or conceivably even a terminally differentiated cell that acquires stem cell properties during oncogenic transformation. For example, both hematopoietic stem cells as well as committed progenitor cells can initiate leukemia after transformation. More recently, activation of canonical Wnt signaling has been shown to transform mouse intestinal stem cells to give rise to adenocarcinomas.

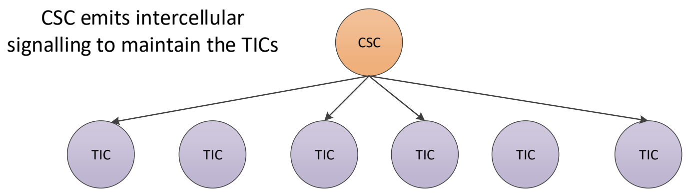
Thus we have as a start three concepts:

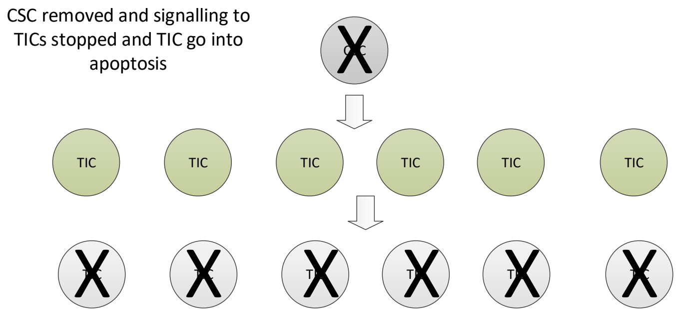
1. Cancer Stem Cell: Also the CSC. This is the self-renewing cell from which the TIC cells arise and which provides the necessary signaling to the TICs to continue their proliferation. Transplanting a CSC will cause a tumor to grow.

2. Tumor Initiating Cell (TIC)[50]: These are cells which are the proliferative cells. They are not the CSC. Transplanting a TIC will result in no growth of a tumor unless accompanied by a CSC.

3. Cell of Origin: Also the Cancer Cell of Origin, CCO. This is the cell from which the CSC was derived. Thus the debate in PCa is often the question; basal or luminal?

This collection leads to a model as shown below:





There is a debate about the existence of CSCs for all cancers as well as how one identifies the CSC if indeed it exists for cancers of specific type. We examine this issue again since in much of the literature there are a multiplicity of definitions.

From Nature we have the following definition[51]:

Cancer stem cells are defined as those cells within a tumour that can self-renew and drive tumorigenesis. Rare cancer stem cells have been isolated from a number of human tumours, including hematopoietic, brain, colon and breast cancers. The cancer stem-cell concept has important implications for cancer therapy. However, the generality of the cancer stem-cell hypothesis has also been challenged…

In a similar manner we have the Tumor initiating cell and its relationship to the CSC and the CCO.

From Agarwal et al we have for TIC:

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[50] Weinberg pp 460-463 discusses the CSC and what he terms the Transit Amplifying/Progenitor Cells.

[51] See: http://www.nature.com/nature/focus/cancerstemcells/

Tumor-initiating cells (TICs), defined by clonal tumor initiation from transplanted cells, have not been analyzed in primary prostate cancers, partly due to the poor transplantation ability of single- cell suspensions of human prostate cancers and low-grade mouse tumors. This may be due to the fragility of fractionated prostate tumor cells, to a high percentage of indolent cells in primary tumors, to a strict requirement for the proper microenvironment, or to other unknown reasons.

Definitions are important. In mathematics and law, the definition will determine the outcome. In engineering we define certain parameters and we design accordingly. If there is a concern that we spend a great deal of time on the definition, that concern should realize that defining something so that it is replicable is a key to scientific study. In cancer studies the term "cancer stem cell" has been introduced but it seems to have been used somewhat loosely.

Definitions should be clear and they should be actionable. Namely the definition should present a way to ascertain through objective measures readily understood by someone trained in the science or art to determine if what is presented satisfies the definition. Namely we should with a good definition know if what we have is a cancer stem cell.

The results below are a sample of what seems to be definitions from the literature. Reading these one can readily see what the complexity is in understanding this topic.

Author	***Definition***
Ailles, and Weissman	*Cancer stem cells (CSCs) are cells that drive tumorigenesis, as well as giving rise to a large population of differentiated progeny that make up the bulk of the tumor, but that lack tumorigenic potential. CSCs have been identified in a variety of human tumors, as assayed by their ability to initiate tumor growth in immunocompromised mice… In addition, specific signaling pathways play a functional role in CSC self-renewal and/or differentiation, and early studies indicate that CSCs are associated with a microenvironmental niche… several important biological properties of CSCs: first, what is the cell of origin for a given tumor? Second, what are the signaling pathways that drive self-renewal and/or differentiation of CSCs? Third, are there molecules uniquely expressed on CSCs, regardless of whether they are functional, that will allow targeted therapies to be developed? Fourth, what are the mechanisms by which CSCs escape conventional therapies and can we defeat these mechanisms?*
Badeux and Tang (in Rajasekhar)	*To fulfill the obligate criteria of a cancer, stem cell (CSC) a cell must be capable of both self-renewal and differentiation, of regenerating and of generating anew…The term cancer stem cell is often replaced by or used synonymously with the phrase tumor initiating cell (TIC).*

Author	***Definition***
Burgess	*Should stem mitotic activity become unregulated or uncontrolled, a tumorigenic and perhaps malignant phenotype may result hence the term cancer stem cell…tumor initiating sells that have malignant properties have been referred to as CSCs…*
Dalerba et al	*Stem cells are defined by three main properties:* *1. differentiation—the ability to give rise to a heterogeneous progeny of cells, which progressively diversify and specialize according to a hierarchical process, constantly replenishing the tissue of short-lived, mature elements;* *2. self-renewal—the ability to form new stem cells with identical, intact potential for proliferation, expansion, and differentiation, thus maintaining the stem cell pool;* *3. homeostatic control—the ability to modulate and balance differentiation and self-renewal according to environmental stimuli and genetic constraints* *Like their normal tissue counterparts, tumors are composed of heterogeneous populations of cells that differ in their apparent state of differentiation. Indeed, the differentiation features of a tumor, morphological and architectural, are the key parameter used in routine clinical practice by surgical pathologists to define a tumor's primary anatomical origin.* *This simple observation suggests that tumors are not mere monoclonal expansions of cells but might actually be akin to "abnormal organs," sustained by a diseased "cancer stem cell" (CSC) population, which is endowed with the ability to self-renew and undergo aberrant differentiation (1, 2). This hypothesis is further reinforced by the fact that cancer is known to result from the accumulation of multiple genetic mutations in a single target cell, sometimes over a period of many years (3). Because stem cells are the only long-lived cells in many tissues, they are the natural candidates in which early transforming mutations may accumulate.*

Author	***Definition***
Dubrovska, A., et al	*One possible explanation for the initial positive response to therapy followed by androgen-refractory disease is that although current therapies eliminate the bulk of the tumor, they fail to eliminate cancer stem cells (CSCs) or tumor-initiating cells (TICs). In fact, it has been argued that many cancers are maintained in a hierarchical organization of rare CSCs, rapidly dividing cells, and differentiated tumor cells; the CSCs are not only a renewable source of tumor cells but are also a source of tumor resistance leading to tumor recurrence, metastasis, and tumor progression. Support for this hypothesis came with the identification of TICs in leukemia in 1994 and, subsequently, in a variety of cancers, including solid tumors. In addition, cancer cell lines have been shown to harbor cancer stem-like cells and are a promising model for CSC research because these progenitors can be readily expanded under anchorage independent (sphere formation) serum-free conditions*
Fang et al,	*Recent studies suggest that cancer can arise from a cancer stem cell (CSC), a tumor-initiating cell that has properties similar to those of stem cells. CSCs have been identified in several malignancies, including those of blood, brain, and breast.*
Hurt et al	*The cancer stem cell hypothesis suggests the existence of a small subpopulation of cells within the tumour that give rise to differentiated tumour cells. It is thought that the cancer stem cells survive conventional treatment to later re-emerge more resistant to therapy. To date, putative cancer stem cells have been identified in blood, brain, breast, lung, skin, pancreas, colon, and prostate*

Author	***Definition***
Jordan et al	*Stem cells have three distinctive properties: self-renewal (i.e., at cell division, one or both daughter cells retain the same biologic properties as the parent cell), the capability to develop into multiple lineages, and the potential to proliferate extensively. The combination of these three properties makes stem cells unique. The attribute of self-renewal is especially notable, because its subversion is highly relevant to oncogenesis and malignancy. Aberrantly increased self-renewal, in combination with the intrinsic growth potential of stem cells, may account for much of what is considered a malignant phenotype. Biologically distinct and relatively rare populations of "tumor-initiating" cells have been identified in cancers of the hematopoietic system, brain, and breast. Cells of this type have the capacity for self-renewal, the potential to develop into any cell in the overall tumor population, and the proliferative ability to drive continued expansion of the population of malignant cells. Accordingly, the properties of tumor-initiating cells closely parallel the three features that define normal stem cells. Malignant cells with these functional properties have been termed "cancer stem cells"*
Lawson and Witte	*Two theories were proposed to explain this paradox. The stochastic theory suggested that all cancer cells are equally malignant but only clones that randomly possess favorable biological properties will grow upon transplantation. An alternative theory predicted that tumors are hierarchical like normal tissues and only the rare subpopulation of cells at the pinnacle of that hierarchy have the unique biological properties necessary for tumor initiation (8, 9). Studies by John Dick and colleagues provided evidence for the hierarchy model. This group demonstrated that only the small subpopulation (0.1%–1.0%) of Lin–CD34+CD38– cells within human acute myelogenous leukemia samples were capable of initiating disease when transplanted into immune-deficient mice (10). These cells possessed the same antigenic profile as normal human HSCs, which are at the pinnacle of the normal hematopoietic hierarchy. This population also had the unique capacity to selfrenew to propagate the disease as well as differentiate to produce the many leukemic cell types represented in the original leukemia. Since these cancer cells possess properties unique to normal tissue stem cells, they have been termed "cancer stem cells" (CSCs).*
Lobo et al	*Stem cell: a primitive cell defined by its capacity to self-renew and differentiate into at least one mature cell type* *Cancer stem cell: a self-renewing cell within a tumor that has the capacity to regenerate the phenotypic diversity of the original tumor*

Author	***Definition***
NCI	*The theory of the cancer stem cell (CSC) has generated as much excitement and optimism as perhaps any area of cancer research over the last decade. Biologically, the theory goes, these cells are distinct from the other cells that form the bulk of a tumor in that they can self-perpetuate and produce progenitor cells, the way that traditional stem cells do. The progenitors' job is then to repopulate tumor cells eradicated by treatments such as chemotherapy or radiation. But for all the attention and fanfare CSC research has received, the findings reported to date are far from clear-cut, investigators acknowledge. For example, most of the studies that have identified human CSCs have used mouse xenograft assays and cells from only a small number of human tumor samples, making it difficult to draw firm conclusions. In addition, other researchers haven't always been able to replicate initially reported findings. And while these tumor-initiating cells, as they are also called, have been described as being a rare class, several studies have found that the number of cells that can form tumors in these mouse experiments is actually quite large, suggesting that perhaps CSCs aren't such a privileged breed.*
Pavlovic and Balint	*As the stem cells that created the tumor to begin with are so few in number, scans following treatment usually fail to identify populations of CSCs in this limited population….*[52]
Perego et al	*Although there is no definitive consensus on the phenotype and frequency of CSCs in the majority of human tumors, much experimental evidence supports the contentions that many tumors of both epithelial and nonepithelial origin have operationally defined CSCs (cells able to propagate tumors in immunodeficient mice) and that the presence of these CSCs affects tumor biology.*
Rajasekhar	*The "cancer stem cell model" CSC …envisions tumors as "pathological organs" sustained in their aberrant growth by a mutated population of stem cells, in which normal homeostatic controls on tissue expansion have been lost.*
Roesch et al	*The CSC concept postulates a unidirectional hierarchy of tumor cells…According to the traditional CSC concept, tumor initiation is regarded as an exclusive characteristic of CSCs*

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[52] This book is near incomprehensible. It is impossible to find a definition, only secondary referral characteristics at best!

Author	***Definition***
Rosen and Jordan	*Thus, the CSC paradigm refers to the ability of a subpopulation of cancer cells to initiate tumorigenesis by undergoing self-renewal and -differentiation, like normal stem cells, whereas the remaining majority of the cells are more "differentiated" and lack these properties.*
Soltysova, et al	*Normal stem cells in the adult organism are responsible for tissue renewal and repair of aged or damaged tissue. A substantial characteristic of stem cells is their ability for self-renewal without loss of proliferation capacity with each cell division. The stem cells are immortal, and rather resistant to action of drugs. They are able to differentiate and form specific types of tissue due to the influence of microenvironmental and some other factors. Stem cells divide asymmetrically producing two daughter cells – one is a new stem cell and the second is progenitor cell, which has the ability for differentiation and proliferation, but not the capability for self-renewal.* *Cancer stem cells are in many aspects similar to the stem cells. It has been proven that tumor cells are heterogeneous comprising rare tumor initiating cells and abundant non-tumor initiating cells. Tumor initiating cells – cancer stem cells have the ability of self-renewal and proliferation, are resistant to drugs, and express typical markers of stem cells. It is not clear whether cancer stem cells originate from normal stem cells in consequence of genetic and epigenetic changes and/or by redifferentiation from somatic tumor cells to the stem-like cells. Probably both mechanisms are involved in the origin of cancer stem cells. Dysregulation of stem cell self-renewal is a likely requirement for the development of cancer. Isolation and identification of cancer stem cells in human tumors and in tumor cell lines has been successful.*

Author	***Definition***
Visvader	*It is important to note that the cell of origin, the normal cell that acquires the first cancer-promoting mutation(s), is not necessarily related to the cancer stem cell (CSC), the cellular subset within the tumour that uniquely sustains malignant growth. That is, the cell-of-origin and CSC concepts refer to cancer-initiating cells and cancer-propagating cells, respectively (Fig. 1). Although the tumourinitiating cell and the CSC have been used interchangeably, the tumour-initiating cell more aptly denotes the cell of origin. There is considerable evidence that several diverse cancers, both leukaemias and solid tumours, are hierarchically organized and sustained by a subpopulation of self-renewing cells that can generate the full repertoire of tumour cells (both tumorigenic and non-tumorigenic cells)1. The cell of origin, the nature of the mutations acquired, and/ or the differentiation potential of the cancer cells are likely to determine whether a cancer follows a CSC model. In most instances, the phenotype of the cell of origin may differ substantially from that of the CSC.* *Normal cellular hierarchy comprising stem cells that progressively generate common and more restricted progenitor cells, yielding all the mature cell types that constitute a particular tissue. Although the cell of origin for a particular tumour could be an early precursor cell such as a common progenitor, the accumulation of further epigenetic mutations by a cell within the aberrant population (in this case expanded) during neoplastic progression may result in the emergence of a CSC. In this model, only the CSCs (and not other tumour cells) are capable of sustaining tumorigenesis. Thus, the cell of origin, in which tumorigenesis is initiated, may be distinct from the CSC, which propagates the tumour.*
Wang and Shen	*In its strictest form, the CSC model posits a hierarchical organization of tumors, with cancer stem cells at the top of the lineage hierarchy being capable of indefinite self-renewal, unlike their progeny, which undergoes an epigenetic program of differentiation and loss of tumorigenicity In this view, rare CSCs may represent the driving force of tumor malignancy, and therefore effective treatment could be achieved by specific targeting of the CSC population. In contrast, the stochastic (clonal) evolution model proposes that most of the cancer cells within a tumor are highly tumorigenic and possess different genetic or epigenetic properties Consequently, it is important to distinguish CSCs that have been strictly defined by their position and function within a lineage hierarchy in vivo from CSCs that have been identified as rare TICs in transplantation studies.*

Author	***Definition***
Weinberg p 462	*…the tumor initiating cell, often termed a cancer stem cell (CSC), is self-renewing and has the ability to generate the countless neoplastic progeny that constitute a tumor. While the CSC and its progeny are genetically identical, the progeny, because they have lost self-renewing ability, have also lost tumor initiating ability.*

It does not take an extensive reading to see the overlap of ideas. Ideas of function and action.

There is often a set of confusion regarding which cell does what. As we have discussed above the CSC is the driving cell for malignant growth. In contrast the CCO is the cell that originally underwent transformation. Is there a connection between them? Clearly the CSC must be some derivative of the CCO. But the CCO is reflective of where the initial genetic alteration occurred. As Tang et al state regarding CSC and CCO we have:

A tumor originates from a normal cell that has undergone tumorigenic transformation as a result of genetic mutations.

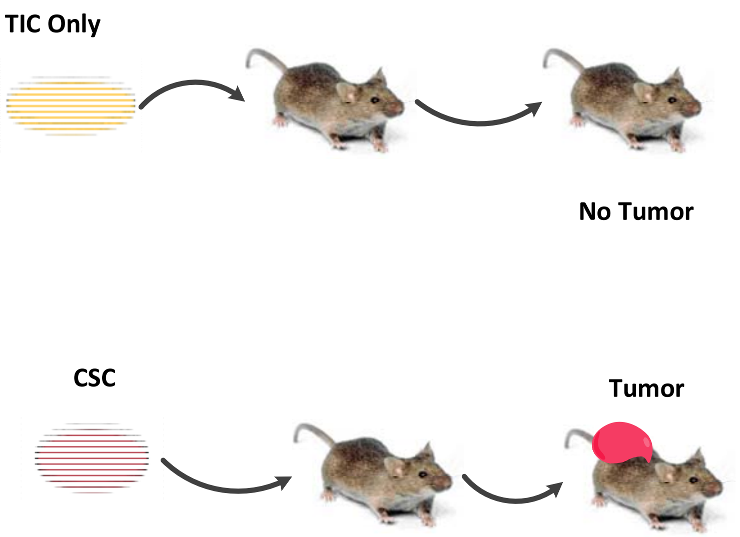
This transformed cell is the cell-of-origin for the tumor.

In contrast, an established clinical tumor is sustained by subpopulations of self-renewing cancer cells operationally called cancer stem cells (CSC) that can generate, intraclonally, both tumorigenic and nontumorigenic cells.

Identifying and characterizing tumor cell-of-origin and CSCs should help elucidate tumor cell heterogeneity, which, in turn, should help understand tumor cell responses to clinical treatments, drug resistance, tumor relapse, and metastatic spread. Both tumor transplantation and lineage-tracing assays have been helpful in characterizing these cancer cell populations, although each system has its strengths and caveats.

In this article, we briefly review and summarize advantages and limitations of both assays in support of a combinatorial approach to accurately define the roles of both cancer-initiating and cancer-propagating cells. As an aside, we also wish to clarify the definitions of cancer cell-of-origin and CSCs, which are often interchangeably used by mistake.

The CCO, cancer cell of origin, is distinct from the CSC. Below we depict a typical test. We select a set of tumor cells. We then mark them with some appropriate marker so that we can separate CSC and TIC cells as well as whatever else is in the mix. The markers are often based on what proteins each cell expresses. The we transplant them to a mouse and examine the result. If we have a CSC, then the tumor regrows. If TIC or benign cells, then no growth.



The above graphic is the approach often used. Namely take a cell which may be expressing a specific surface marker and then implant it in a mouse and observe the result. If the cell replicates the human tumor, then we have "found" the CSC. It is not clear that mice may not be primed for this. It is not clear how coincidental this may be. There should be a body of justification which is much more extensive.

8.4 A THOUGHT EXPERIMENT

The cancer stem cell concept is somewhat akin to the overall stem cell. Simply, a Cancer Stem Cell appears to be as a concept a single stem cell with some well-defined DNA structure which becomes capable during mitosis of;

(i) regenerating itself consistently in some near immortal manner,

(ii) while simultaneously generating another cell which is different from itself and which itself may duplicate itself exactly, subject to random genetic changes, and

(iii) that such CSC if transplanted alone to some unaffected carrier will regenerate the tumor from which it was extracted.

This definition is an amalgam of the many attempts to define such a cell.

We know that such a process as the stem cell, albeit benign, appears to exist in hematopoiesis. Also it has been argued that such a cell is the basis for a variety of hematopoietic malignancies, such as MDS. MDS is especially interesting since it occurs not with the hematopoietic stem cell but somewhere along the line such a myelo or lympho line and that it involves methylation yet there is a CSC like behavior.

Let us begin with some facts:

1. All somatic cells have the same DNA. This is almost true. There are exceptions as follows:

a. There may have been some somatic mutation or translocation.

b. There may be some epigenetic changes due to methylation or miRNAs for example.

2. Mitosis of a single cell produces two identical offspring. There are some differences however:

a. First what do we mean by identical? They clearly have the same DNA but some DNA may be expressed slightly differently. Why is one cell expressing DNA differently than the other? Why is the other cell, if that be the case, working identically as its parent cell? Are the previous statements true?

b. Phenotypically there may be a significant difference in the cells.

3. A stem cell is defined in a certain manner. Essentially it is a self-replicating cell that can give rise to itself by definition and to other cells which may become mature cells in some terminal sense. However:

a. How does one identify a stem cell? Generally, it has been identified as a cell which when transplanted to a genetically primed target, a mouse for example, that it generates and reproduces the initial cancer. Furthermore, if it is silenced or removed the cancer ceases.

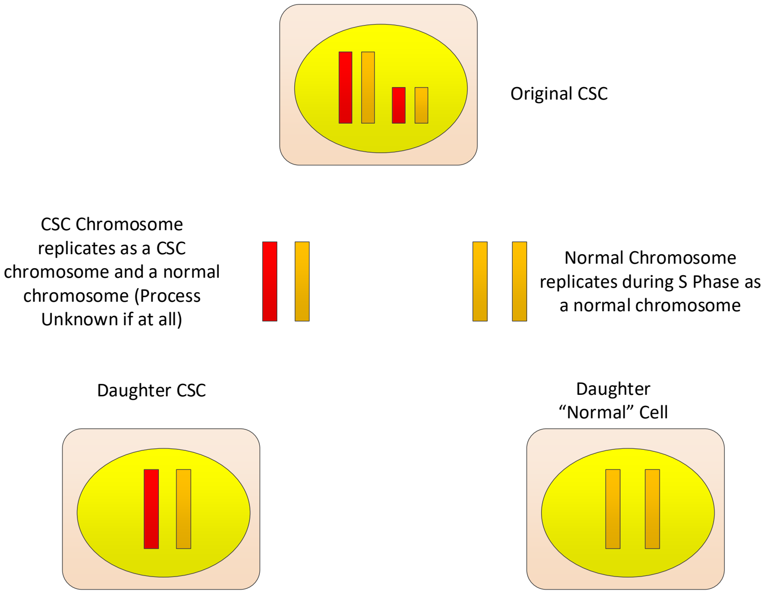
4. A cell of origin is a cell from which the original cancer arises. Yet:

a. What do we mean by the original cancer?

b. What is the relationship to the CSC?

5. A cancer stem cell, CSC, is a cell which can be defined as a self-replicating cell which also produces a second type of cell which is less self-replicating but which becomes the body of a tumor. The CSC somehow using the same DNA manages to go through the cell cycle yet produces two phenotypical cells which are also genotypically different in their expression albeit genotypically the same in toto.

We try to demonstrate this artifact below. One must note that there is as of yet no physical basis for this claim. It is merely a thought experiment.



What do we have above? We have the following:

1. A cell with DNA that has somehow had some malignant alteration in two of the chromosomes. We have one chromosome marked as red which renders some semblance of immortality and a second chromosome which is marked orange which renders excess growth, albeit with limited mortality. Homologous orange chromosome cells are aggressive growers but can die off.

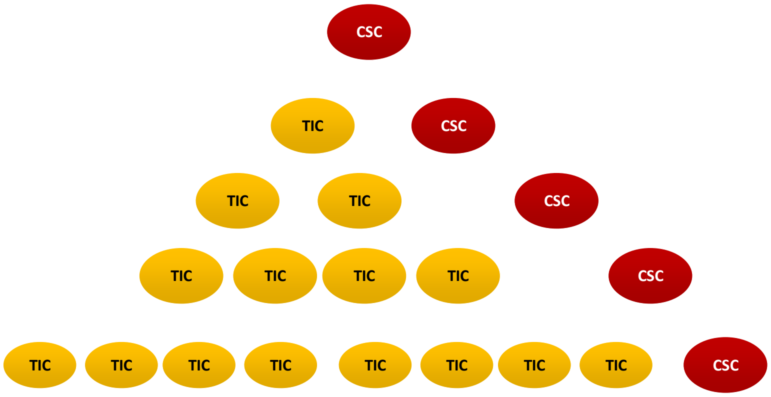
2. Now somehow, we really do not know but just posit a result, which makes this a thought experiment, the CSC goes through mitosis and produces two cells; a duplicate of itself and a daughter cell with homologous orange chromosomes.

3. The homologous cell goes on to replicate and then can go into apoptosis and die off.

4. The CSC can replicate again.

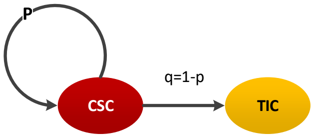
5. The CSC can replicate in one of two ways. First it can be deterministic, namely one CSC yields one CSC and a homologous cell. Second one CSC can regenerate itself with some probability and produce a homologous cell with another probability. The latter is the stochastic case.

We demonstrate the deterministic below:



Deterministic: The CSC replicates itself each time and the TIC also replicates but it doubles. Thus we see a single CSC while the TICs double.

We demonstrate the stochastic as follows:



Stochastic: The CSC can split into another CSC with a probability p or a TIC with probability q. If, for example, it splits into 2 TIC then the CSC could die off. It also could split into two CSC which would then cause added growth.

With the above model one can determine the distribution of cells as a function of time. For a linear progression the split is always 50:50 and otherwise we would have a probability that the CSC itself could extinguish. Even if p approaches 1.0. and never really reaches it then there is a minute but possible extinction. We do have examples of tumor regression. The classic case is in melanoma and in Rosenberg's early observations. We also know that in the case of HGPIN, that most likely we have some form of stem cell and that HGPIN also regresses in a finite number of cases[53].

A great deal of work has been done examining the dynamics of CSCs. Part of that efforts pertains to establishing some means to identify them. We examine a small subset of the models here but there are many studies worth examining. Fundamentally the studies all seem to reflect the approach which starts with a stem cell and examines the products resulting therefrom. We

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[53] It is worth reading some of the cases we have discussed in the White Papers. There is a recent case where we saw total extinction.

have argued elsewhere and summarize latter herein that there is an alternative approach which frankly eschews the CSC and examines a collection of cells each sub-collection having a specific genetic expression state. That approach only looks at the cancer as a separate organism from the host and tries to understand it holistically as a spatio-temporal collection of interdependent genetic expression states evolving over time.

Many authors have examined the mathematical dynamics of the stem cell and the CSC. Stukalin et al have developed models for the fluctuations in cell populations. In a sense this is always a significant issue since the CSC growth is complex and does not reflect a simple deterministic model. Dhawan et al have examined the tumor control mechanisms in dynamic CSC environments. This is one of many ways that the CSM paradigm could be used in the control of cancers. Shahriyari et al have examined mathematical models for the stochastic dynamics of the CSC environment. They look at multiple mutations and effects on non-symmetric changes. Zhang and Wolynes use the many-body paradigm to explore stability points in complex CSC models. These are but a few of the approaches taken in modelling the CSC environment.

8.5 SOME BASICS

Let us begin with some simple fundamentals. As Dingli and Pacheco note:

Tissues have evolved an architecture where most cells have a relatively short lifetime and undergo continuous turnover, and this mitigates the accumulation and retention of mutant cells.

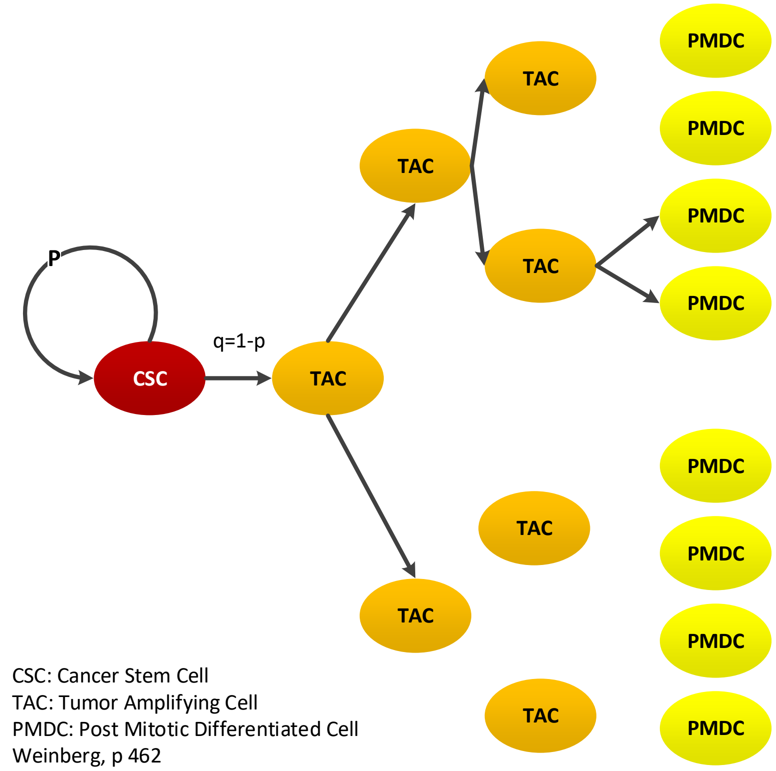
At the root of this process are the stem cells that are able to maintain tissue integrity because of a dual phenotypic characteristic: self-renewal and production of progeny that can differentiate into various cell lineages that together constitute tissues and organs.

One can visualize tissues as having a tree-like organization of cells with stem cells at one extreme and mature, non-dividing cells at the other extreme.

Intermediate cells divide, often at relatively high rates, but live for relatively short periods of time. Although mutations can occur at every level of this cell hierarchy, the relatively short lifetime of more mature cell stages means that, in effect, the real risk of long-lasting oncogenic mutations is restricted to the small population of stem cells and early progenitor cells that maintain a given tissue.

This, in turn, effectively reduces the probability of the occurrence of mutations, given the small population of cells at risk, despite the fact that a mutation arising in a stem cell can persist for a long time. It is important to point out that the relevance of a mutation is cell context-dependent – a mutation in a gene that is not expressed in a cell is of no consequence to that cell but expression of the gene in more committed cells, downstream of the cell that is the source of the mutation, may lead to a phenotype associated with disease.

From Weinberg we have the following model which reflects the above:



CSC: Cancer Stem Cell
TAC: Tumor Amplifying Cell
PMDC: Post Mitotic Differentiated Cell
Weinberg, p 462

Note that in the above model we have the long lasting CSC and then we have proliferating intermediaries and ultimately the non-proliferating end stage cells. As the above authors note that since this is stochastic then there is a multiplicity of end states. At one extreme the CSC may actually die off, it may not reproduce and thus the cancer may just regress. We have argued that in certain cases of HGPIN followed by high saturation prostate biopsy that one may actually capture the CSC in a single core and thus deprive the nascent malignancy of its growth potential. Also one could imagine the immune system performing a similar function.

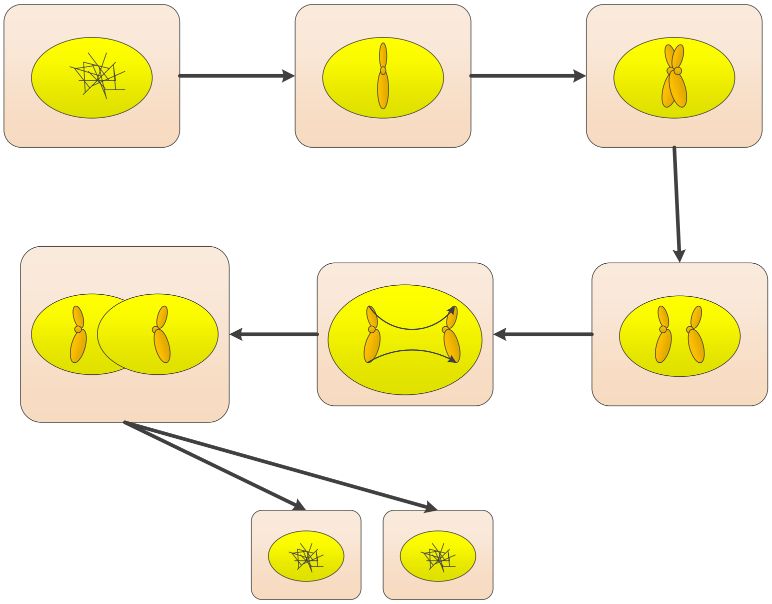
Bogdan et al report:

Stem cell division times exhibit non-stationary behavior. Besides the heterogeneous structure of stem cells population, we also observe that the empirical PDF estimated from stem cell DTs exhibits a pronounced time dependent behavior…

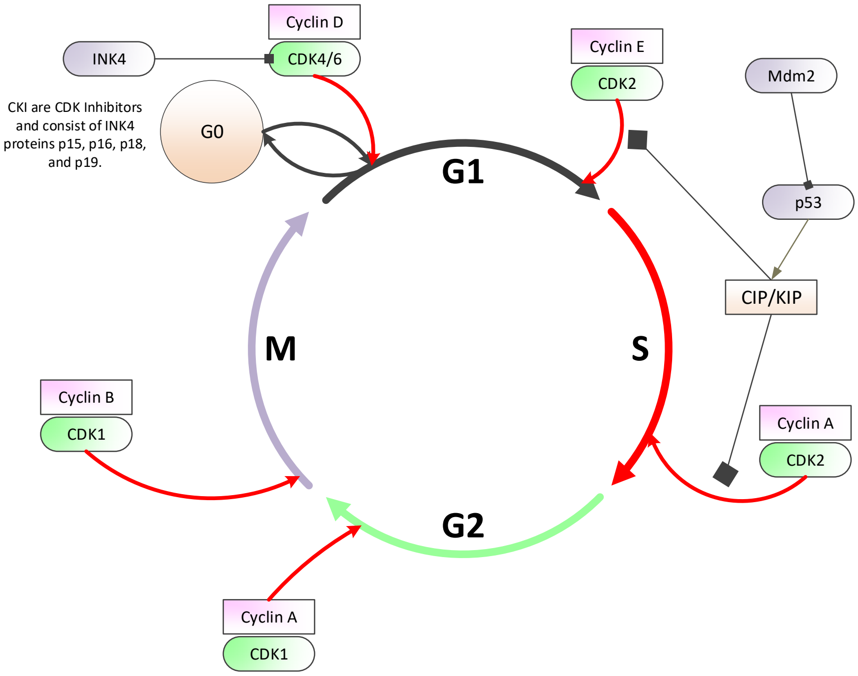
Stem cell growth rates possesses multi-fractal characteristics. For a comprehensive investigation of the heteroscedastic dynamics of stem cell growth, we investigate the relationship between the higher order moments of stem cells dynamics and their order; we also estimate both the multi-fractal spectrum and generalized Hurst exponent function

8.6 THE CELL CYCLE

A fundamental element of the understanding of cancer dynamics and the issues related to CSCs is the cell cycle itself. We start with a simplified description of mitosis. The intent here is not to present mitosis which is well documented in a multiplicity of places but to place a focus on some of the issues of the CSC. The simplified cycle is below:



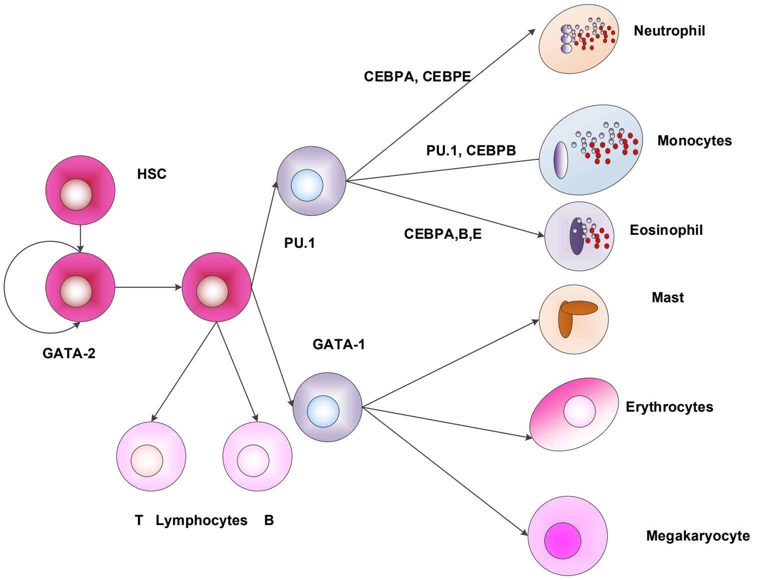
Now note the key step is in the reproduction or duplication of the DNA in the S phase. That is the last step on the top, we see a doubling of the chromosome. We detail the cycle below for reference.



Now let us consider two processes in which this occurs:

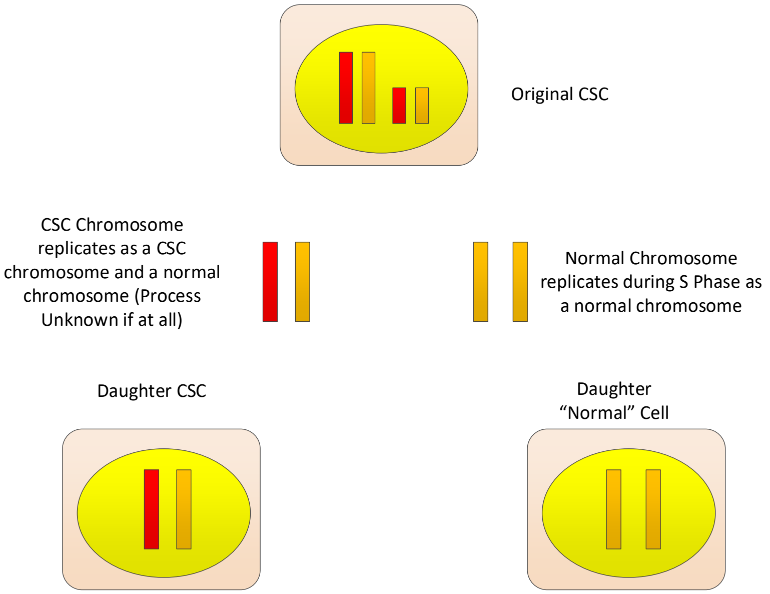
1. Hematopoiesis: As the stem cell for the various blood lines evolve as shown below we have a cell move along but it changes based on what its local environment presents. The stem cell in the

bone produces two stem cells, one which stays put, I am assuming a deterministic model, and another moves, and as it moves it encounters ligands that attach to receptors and the cell begins to change. As it changes it goes through mitosis again perhaps and it again encounters more ligands and changes some more.



This process continues until complete maturity.

2. CSC: This model is problematic Recall the assumption below:



There is the issue of recreating the CSC while also creating a new cell where the S phase appears to have some asymmetry. This is problematic. There is no well-known process whereby this can occur.

8.7 IDENTIFYING CSCS

There are currently several ways to identify CSCs. The primary one is via cell surface markers and in the case of PCa one specific one is CD44. Karsten and Goletz present a recent review of a collection of such markers. As they note:

In recent years' considerable effort has been invested in the detection and characterization of stem cell markers. The result is that there are now an overwhelming and steadily increasing number of such marker molecules. Some markers are indeed more or less specific for different types of stem cells, for example, markers that differentiate embryonic from adult stem cells or pluripotent from progenitor cells. With the exception of pluripotent embryonic stem cells all other stem cells carry, in addition, lineage-specific markers.

Stem cells are also defined by the absence of certain markers. Contemplating these data, several questions arise. First, as already mentioned, almost all markers of normal stem cells are also found on cancer stem cells. This, of course, poses a problem with respect to their potential use as therapeutic targets. Ectopic (non-lineage) expression of stem cell markers on cancer cells does not resolve the therapeutic dilemma. Currently the best option for a therapeutic target would be to rely on onco-fetal stem cell markers which are not expressed on normal adult stem cells. Otherwise there is at present no clear-cut distinction available between normal and cancer stem cell markers. Even at the level of regulatory miRNA clusters, identical patterns were observed

They continue:

These data and other more general considerations led us to propose the following hypothesis.

1. During the process of malignant transformation from a normal stem or progenitor cell to a cancer stem cell, stem cell glycoprotein markers undergo alterations in their glycosylation.

2. As a consequence, cancer stem cells carry cancer specific glycans.

3. This appears to be a selective process. Accordingly, these cancer-specific glycans are CSC makers.

4. Changes in stem cell marker glycosylation contribute to the altered biological behavior of these cells.

In brief, we propose that cancer stem cell markers differ from their normal counterparts by the expression of tumor-specific glycans.

We have seen the glycan presence previously. But the change in glycosylation may be a change in energy utilization which we have also seen in the Warburg process. Thus the glycan markers may logically be targeted as markers. The logic and data in this paper may add more to the understanding of the CSC dynamics.

8.8 PATHWAY ISSUES

We briefly examine some of the key pathways that have been argued as critical in the CSC evolution. Although we present them we however do not attribute anything specific to them herein. As Zhang et al note:

IPA uncovered important signaling pathways enriched in basal cells including

1. *TGF-b,*
2. *NOTCH,*
3. *WNT/TCF,*
4. *IGF,*
5. *FGF,*
6. *STAT3/ IL6 and others.*

For instance, immunofluorescence of FGFR3 validated our RNA-Seq data and revealed its expression preferentially in the basal layer. We systematically investigated some of these pathways in regulating primary basal stem/progenitor activities.

Given that each pathway has a large number of components, we first used the pathway-specific pharmacological inhibitors to interrogate their roles in regulating basal cell activity. For pathways of particular interest, small interfering RNA (siRNA)-mediated knock-down experiments were performed to validate the inhibitor results.

8.8.1 WNT

We briefly re-examine each of these. First we show the WNT pathways below. This is a well know process and we have examined it extensively previously[54].

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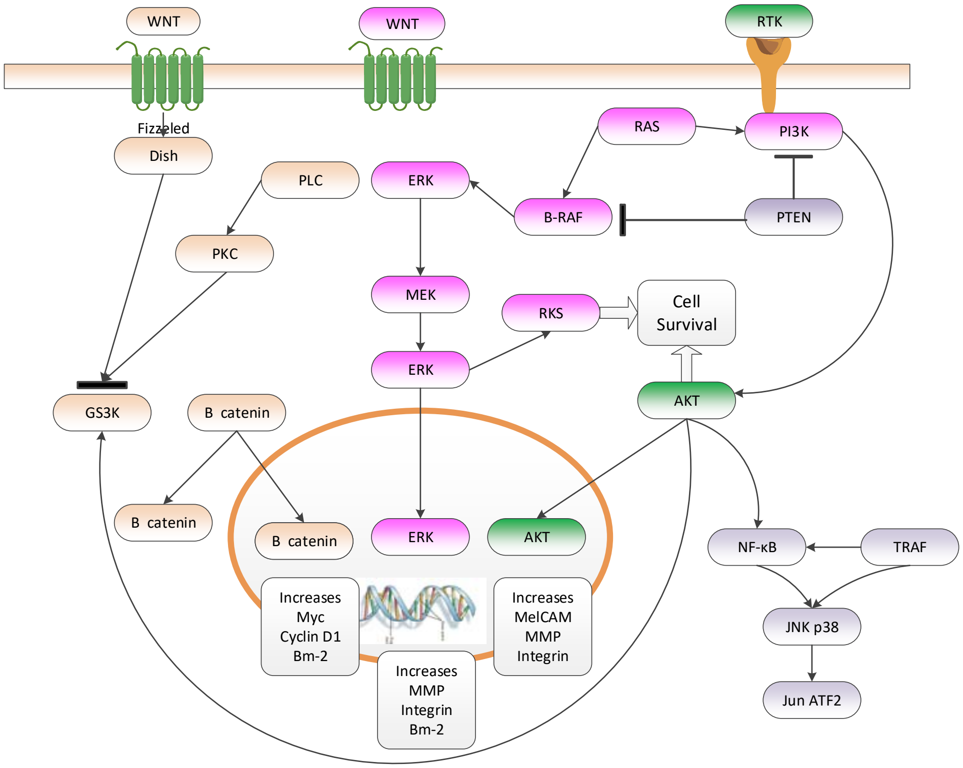
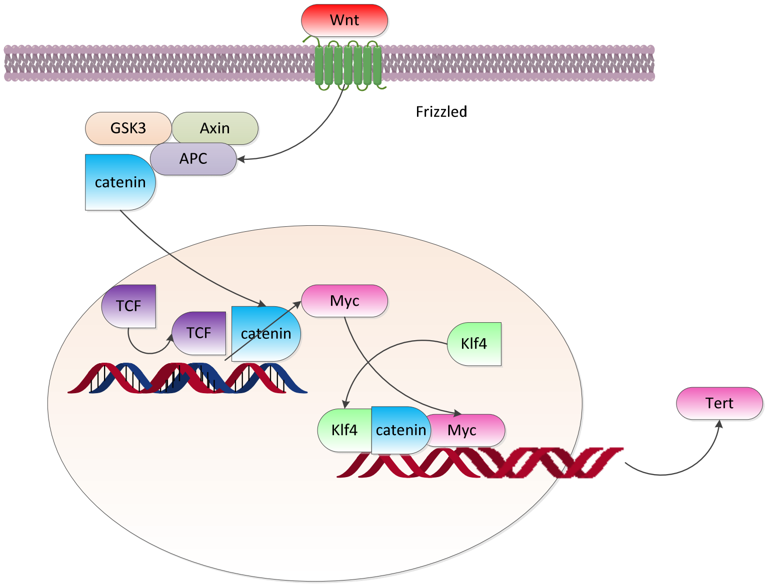
[54] Specifically see the reference by Goss and Kahn.



Signaling pathways in the cells have been a major focus on study for the past decade or so. The focus generally has been on what protein or gene influences what other protein or gene. A recent article in Science presents some interesting work on Wnt and TERT[55].



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[55] http://science.sciencemag.org/content/336/6088/1519

Wnt is an extra cellular signaling protein and it attaches to Frizzled a receptor and sets off a cascade that moves B catenin into the nucleus and generates Myc which is a transcription protein with together with catenin and other transcription proteins generates Tert from TERT.

To quote from NCBI[56]:

Telomerase is a ribonucleoprotein polymerase that maintains telomere ends by addition of the telomere repeat TTAGGG. The enzyme consists of a protein component with reverse transcriptase activity, encoded by this gene, and an RNA component which serves as a template for the telomere repeat. Telomerase expression plays a role in cellular senescence, as it is normally repressed in postnatal somatic cells resulting in progressive shortening of telomeres. Deregulation of telomerase expression in somatic cells may be involved in oncogenesis.

As the Science article states:

Maintaining the length of telomere, the ends of chromosomes, is essential for all cells that divide many times. The enzyme telomerase lengthens these ends, counterbalancing their shortening that occurs each time chromosomes are copied. Telomerase is essential for cell viability, and loss of its function from the loss of only one of two copies of the encoding gene can lead to the failure of stem cell renewal that is seen in premature aging conditions such as dyskeratosis congenita, aplastic anemia, and pulmonary fibrosis. Conversely, telomerase activity is increased in many cancers and may be required for cancer cells to maintain their telomere length...

They continue is a rather interesting wording:

Because of the importance of telomerase expression, the signaling pathways that control TERT transcription have been extensively studied. Remarkably, many different transcription factors, including c-Myc, Sp1, nuclear factor of activated T cells (NFAT), activating protein 2B, nuclear factor κB (NF-κB), Myb, activating transcription factor, nuclear factor 1 (NF1), and the estrogen receptor (ER), bind to the 330–base pair minimal TERT promoter and regulate transcription. In addition, a number of negative regulators bind the TERT promoter, including CTCF, elongation factor 2, p53, Ets, Mad1, Men1, and Wt1. Adding β-catenin and Klf4 to the many regulators that bind the TERT promoter is like adding one more guest to a crowded table at a dinner party.

They conclude:

It is reasonable to propose that Wnt regulates TERT given that Wnt signaling plays an essential role in stem cell self-renewal and that TERT is needed for the long-term growth of stem cells. TERT regulation seems to require not one, but two master transcriptional regulators to assure that there is neither too much, which may allow the growth of cancer cells, nor too little, which might lead to stem cell failure. The finding by Hoffmeyer et al. that both β-catenin and Klf4 are required to activate TERT expression puts the horse (Wnt) before the cart (TERT) and provides a foundation for linking telomerase levels and self-renewal.

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[56] http://www.ncbi.nlm.nih.gov/gene/7015

The observation of the inter-cellular signaling with Wnt and its control over TERT and the telomere process is quite interesting. This may be an interesting way to incorporate many of the Turing models we have been discussing as well.

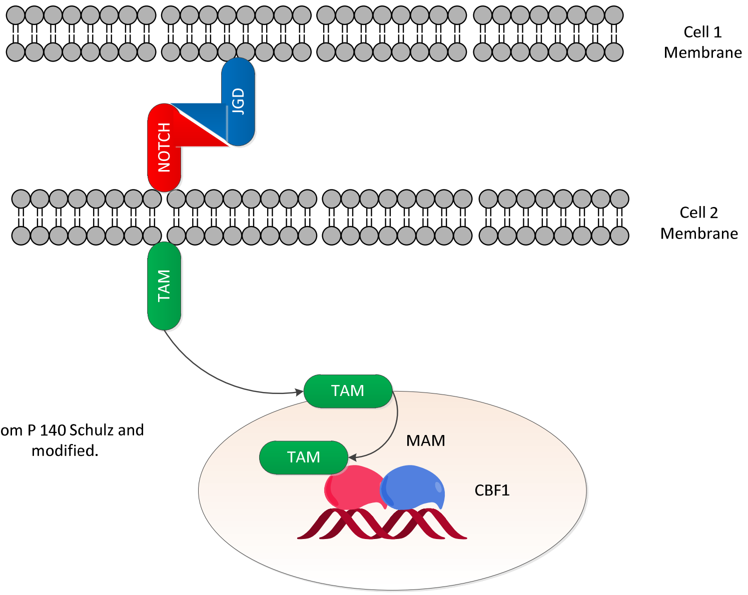
8.8.2 NOTCH

Notched is a bit of an amalgam of the above discussion. The notched pathway is characterized as follows.

The notch protein sits like a trigger spanning the cell membrane, with part of it inside and part outside. Ligand proteins binding to the extracellular domain induce proteolytic cleavage and release of the intracellular domain, which enters the cell nucleus to alter gene expression. The notch signaling pathway is important for cell-cell communication, which involves gene regulation mechanisms that control multiple cell differentiation processes during embryonic and adult life. Notch signaling also has a role in the following processes:

1. neuronal function and development
2. stabilization of arterial endothelial fate and angiogenesis
3. regulation of crucial cell communication events between endocardium and myocardium during both the formation of the valve primordial and ventricular development and differentiation
4. cardiac valve homeostasis, as well as implications in other human disorders involving the cardiovascular system
5. timely cell lineage specification of both endocrine and exocrine pancreas
6. influencing of binary fate decisions of cells that must choose between the secretory and absorptive lineages in the gut
7. expansion of the hematopoietic stem cell compartment during bone development and participation in commitment to the osteoblastic lineage, suggesting a potential therapeutic role for notch in bone regeneration and osteoporosis
8. T cell lineage commitment from common lymphoid precursor
9. regulation of cell-fate decision in mammary glands at several distinct development stages
10. possibly some non-nuclear mechanisms, such as control of the actin cytoskeleton through the tyrosine kinase Ab

We demonstrate Notched and its counterpart Jagged in the following Figure. On the cell surface we have Notched and on the other cell surface we have Jagged. When they bond, in a sense as surface proteins but with a communicating capability, Notched release or activates Tam which is a transcription factor facilitator.



From P 140 Schulz and modified.

Notch signaling is dysregulated in many cancers.

8.8.3 FGF

FGFR is a Receptor and this gene encodes a member of the fibroblast growth factor receptor (FGFR) family, with its amino acid sequence being highly conserved between members and among divergent species. FGFR family members differ from one another in their ligand affinities and tissue distribution. A full-length representative protein would consist of an extracellular region, composed of three immunoglobulin-like domains, a single hydrophobic membrane-spanning segment and a cytoplasmic tyrosine kinase domain. The extracellular portion of the protein interacts with fibroblast growth factors, setting in motion a cascade of downstream signals, ultimately influencing mitogenesis and differentiation. This particular family member binds acidic and basic fibroblast growth hormone and plays a role in bone development and maintenance.

As we have noted elsewhere FGF is one of many such receptors as shown below:

Models	*Genes regulated*	*Prostate phenotype*
Hormone receptors	Androgen receptor	HGPIN
	Retinoic acid receptor α/γ	Squamous metaplasia and pre-neoplastic lesions
	Estrogen receptorα/β	No marked phenotype
Growth factors and receptors	FGF8b	HGPIN
	FGFreceptor1	PIN with reversible hyperplasia
	FGF7	Prostate epithelial dysplasia
	FGFR2iiib	Hyperplasia/dysplasia
	IGF-1	PIN and spontaneous tumor growth
	TGFR-β	PIN and invasive adenocarcinoma
	HER-2/Neu	PIN and invasive carcinoma
Tumor suppressors, cell cycle, and signaling pathways	p53Rb	PIN with reduced apoptotic potential Focal hyperplasia
	Nkx3.1	Hyperplasia followed by PIN
	H-Ras	LGPIN and intestinal metaplasia
	APC	PIN and invasive adenocarcinoma
	Pten	PIN and metastatic adenocarcinoma
	Bcl-2	No overt phenotype
	Akt-1	Focal regions of PIN
	C-MYC	PIN and locally invasive adenocarcinoma
Genomic instability	Eco RI c-fos	HGPIN No significant pathology
Composite transgenic mice	Ink4a/Arf+/−/Pten +/−	Rapid growth of PIN lesion
	Nkx3.1/Pten	PIN and metastatic spread of invasive tumors to lymph nodes
	Pten+/−/Akt1−/−	Akt1−/− repressed prostate tumor growth
	Pten+/−/p27kip1−/−	Rapid progression of invasive carcinoma
	Pten−/−/p53−/−	Early onset of invasive tumors
	PTEN+/−/TRAMP	Increased rate of tumor development
	P53−/−/Rb−/−	Highly metastatic adenocarcinoma
	Pten+/−/FGF8b	Metastatic adenocarcinoma
	Bcl-2/TRAMP	Multi step prostate carcinogenesis

8.8.4 TGF-β/EMT

As we had noted previously[57]:

TGFB1: This gene encodes a member of the transforming growth factor beta (TGFB) family of cytokines, which are multifunctional peptides that regulate proliferation, differentiation, adhesion, migration, and other functions in many cell types.

Many cells have TGFB receptors, and the protein positively and negatively regulates many other growth factors. The secreted protein is cleaved into a latency associated peptide (LAP) and a mature TGFB1 peptide, and is found in either a latent form composed of a TGFB1 homodimer, a LAP homodimer, and a latent TGFB1-binding protein, or in an active form composed of a TGFB1 homodimer.

The mature peptide may also form heterodimers with other TGFB family members. This gene is frequently upregulated in tumor cells, and mutations in this gene result in Camurati-Engelmann disease.

We now focus on the issue of prostate cancer, PCa, and its stem cell as well as its cancer cell of origin, CSC and CCO.

The prostate is an interesting organ. It is a collection of glandular cell segments and the glands contain a circumference of basal cells and a collection of luminal cells extending into the lumen, the empty space. Around the periphery and in the connective space are a multiplicity of other cells of various types; muscle cells, neuroendocrine cells and the like. The prostate tends to grow or enlarge as a man gets older and thus can grow from a typical size of 40 cc to at times well over 100 cc. In simple age related benign growth the prostate duplicates itself in an ever enlarging glandular network and generally appears somewhat uniform. Inflammation may occur as well as hyperplasia.

The hyperplasia generally appears as masses of excess and somewhat disordered luminal cells in the lumen, and the organization of the lumen begins to become distorted. In the extreme case of High Grade Prostatic Intraepithelial Hyperplasia, HGPIN, the gland appears almost filled with luminal cells. Some have argued that this is a precancerous state and irreversible. We however have seen cases where it is totally reversible and thus this existence proof of non-inevitability is questionable.

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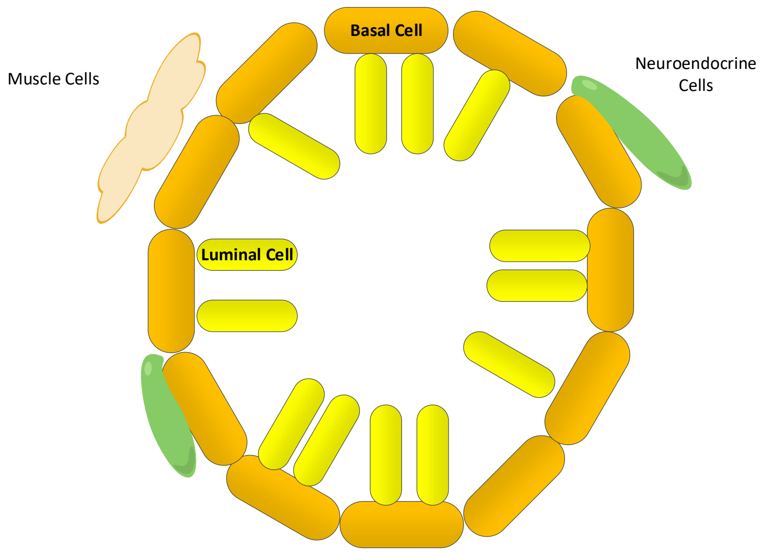
[57] See White Paper No. 133 LY6 and Prognostic Markers (February 2016)



As Agarwal et al note:

Prostate glands are composed of:

1. *an outer layer of basal cells expressing KRT5, KRT14, and TP63,*
2. *an inner layer of secretory, luminal cells expressing KRT8, KRT18, and AR,*
3. *and rare SYP and CHGA positive neuroendocrine cells.*

TP63 is a marker of prostate basal epithelial and stem cells and is required for prostate development. Lineage tracing studies based upon cytokeratin drivers have established a number of principles for stem cell hierarchies in the developing and adult prostate.

The majority of regenerative adult stem cells appear to be unipotent. In addition, studies using other lineage tracing schemes have described minor populations of multipotent progenitor cells that have not been captured with KRT-specific drivers.

Using an inducible NKX3.1-specific CRE driver, a rare (0.7%) population of bipotential luminal cells in the castrate prostate (CARNs) has been described (Wang et al., 2009). In addition, the existence of KRT5neg, KRT14-,TP63+ cells has been observed, as well as the ability of TP63 lineage marked cells to generate luminal epithelial cells in the adult

As PCa develops it initially appears as a multiplicity of poorly formed glandular structures, and although looking somewhat like the benign normal glands it starts to loose structure as it develops. The question than is; what cells is the basis for this change, basal or luminal or other, and as the PCa starts to expand which cell is and/or becomes a CSC?

From White and Lowry, we have a summary of the issue regarding the cell of origin for PCa. They note:

Models for both murine and human prostate cancers have produced conflicting conclusions within the field as to whether the CCO is of basal or luminal origin. Debate has arisen as to whether the stem cells of the prostate reside in either the basal or luminal populations.

Using a broader range of lineage tracing alleles, it was suggested that a multipotent population arises from the basal population, while separate unipotent progenitors populate the neuroendocrine and luminal pools. The lack of a consensus on the identity of ASCs of the prostate has also clouded the interpretation of CCO studies for the prostate. Similar to the discrepancies observed for SCC/BCC, much of the debate regarding CCOs for prostate cancer centers on the fact that prostate tumors typically adopt a morphology consistent with a luminal origin, while experimental data often point towards a basal source for CCOs.

Human prostatic epithelial transplantation studies, which do not include a native stromal and immune component, indicated a basal CCO with MYC, AKT or ERG as oncogenic drivers. By contrast, genetically modified mouse models that used Pten deletion implicated both basal and luminal cells as CCOs, depending on the targeting alleles and tumorigenic strategies used.

In addition, one study showed that initiation from human basal cells generates transformed luminal-like cells that are able to propagate the tumor. Together, these results suggest that the identity of the CCO for prostate cancer could be dependent on cellular, genetic, and environmental contexts, and further work will be needed to address whether differences exist between human and mouse models systems or whether the differences are caused by nonequivalent cell-intrinsic and cell-extrinsic stimuli.

Heterogeneity of tumor initiators and tumor phenotypes The experimental models described here have proven to yield important insights into tumor initiation and CCOs. However, there are technical limitations to these models that ignore the heterogeneity of bona fide cancer initiation. Tumors are thought to be initiated in a clonal fashion as a result of mutations.

We thus examine this CCO and corresponding CSC issue for PCa.

The Cell of Origin is akin to the CSC and has been the focus of debate in PCa. As White and Lowry have noted:

Significant progress has been made to identify the cells at the foundation of tumorigenesis, the **cancer cell of origin (CCO).** *The majority of data points towards resident* **adult stem cells (ASCs) or primitive progenitors as the CCO for those cancers studied, highlighting the importance of stem cells not only as propagators but also as initiators of cancer**. *Recent data suggest tumor initiation at the CCOs can be regulated through both intrinsic and extrinsic signals and that the identity of the CCOs and their propensity to initiate tumorigenesis is context dependent. In this review, we summarize some of the recent findings regarding CCOs and solid tumor initiation and highlight its relation with bona fide human cancer.*

Cancer is a complex disease due to the wide variety of cellular and molecular mechanisms associated with its initiation and progression. It is accepted that cancer cells divide and proliferate uncontrollably because of the accumulation of somatic mutations in normal tissue, which confers a selective growth advantage in the mutated progeny.

However, the cells that make up a tumor are heterogeneous; often making it difficult to determine the ***CCO, which is the normal cell that acquires the mutational load necessary to first initiate cancerous proliferation****. Furthermore, since cancer is a transformative process, the cells composing advanced cancers may no longer contain morphological or molecular characteristics of the CCO. The identity of the CCO could be critical to the generation of more effective treatments and preventative strategies.*

If CCOs can be identified and targeted specifically, it would be possible to stop cancer before it has a chance to undergo expansion. Molecular or physiological attributes specific to CCOs could be exploited to slow or block progression, thus avoiding treatments that simply kill dividing cells. This has led to significant recent efforts to define CCOs for all types of cancers, and numerous lines of evidence point towards ASCs as possible CCOs.

They continue:

ASCs are found in many of the major adult organs and are essential for tissue homeostasis as well as regeneration in response to injury*.*

Most ASCs were discovered on the basis of their relative quiescence and their ability to reconstitute differentiated cell lineages of the tissue or organ in which they. Either upon activation by natural turnover/cycling or in the case of regeneration due to injury, ***ASCs give rise to multilineage restricted progenitors or, as they are often called, transit amplifying cells (TACs).***

These cells divide rapidly and then differentiate to generate the bulk of cells required for tissue turnover or regeneration. Due to their rapid division, TACs are also targeted by chemotherapeutics that act on cell division pathways to kill cancer cells

In the above we have seen defined three entities:

1. CCO: The cancer cell of origin.

2. ASC: Adult stem cells.

3. TAC: Transit amplifying cells

Wang and Shen note:

A similar confusion arises with respect to the cell of origin for cancer, which corresponds to a normal tissue cell that is the target for the initiating events of tumorigenesis. In principle, a normal adult stem cell could be a logical cell of origin for cancer, as it would retain the ability to self-renew and generate a hierarchy of differentiated lineages within a tumor. However, it is also possible that a cell of origin could correspond to a downstream progenitor cell or conceivably even a terminally differentiated cell that acquires stem cell properties during oncogenic transformation.

Thus we even here have some confusion as to the CCO, cancer cell of origin.

One of the sets of arguments presents the basal cell as the cell of origin. As Wang and Shen note:

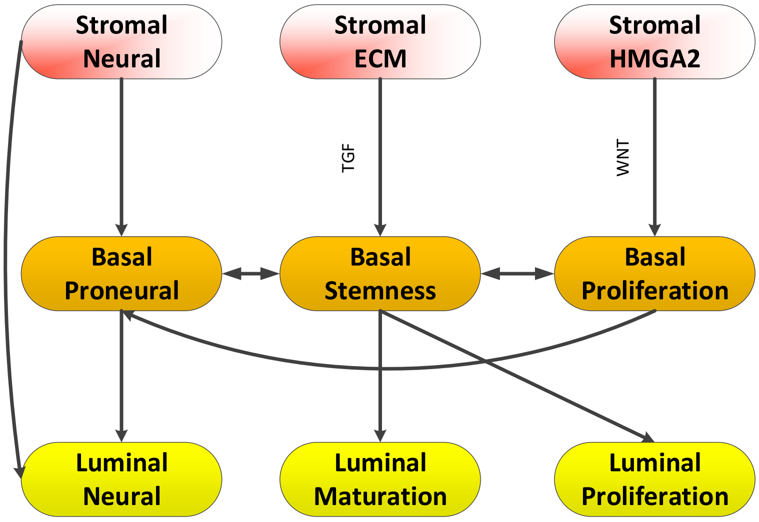
Although prostate tumors display a strongly luminal phenotype, this does not exclude the possibility that basal cells could be a cell of origin for prostate cancer. In particular, it is possible that transformed basal cells could differentiate to generate large numbers of luminal cancer cells. For example, prostate-specific conditional deletion of Pten by a probasin-Cre driver allele has been shown to result in a basal cell expansion accompanied by increased number of intermediate cells, suggesting a basal cell of origin.

In a paper by Goldstein et al they note:

Luminal cells are believed to be the cells of origin for human prostate cancer, because the disease is characterized by luminal cell expansion and the absence of basal cells. Yet functional studies addressing the origin of human prostate cancer have not previously been reported because of a lack of relevant in vivo human models. Here we show that basal cells from primary benign human prostate tissue can initiate prostate cancer in immunodeficient mice.

The cooperative effects of AKT, ERG, and androgen receptor in basal cells recapitulated the histological and molecular features of human prostate cancer, with loss of basal cells and expansion of luminal cells expressing prostate-specific antigen and alpha-methylacyl-CoA racemase. Our results demonstrate that histological characterization of cancers does not necessarily correlate with the cellular origins of the disease.

We had examined this in some detail when it first appeared some six years ago. The problems were that it was murine related and one could argue that CSC in a mouse is not CSC in human. Suggestive but not a definitive proof.



From Moscatelli and Wilson we have the arguments:

In a recent paper in Science (3), Goldstein et al. describe a model system in which questions about the cell of origin and oncogenic pathways of human prostate cancers can be addressed. Using two cell surface antigens, Trop2 (TACSTD2) and CD49f (integrin α6), Goldstein et al. (3) separated luminal (Trop2+/CD49f−) from basal (Trop2+/ CD49f+) cells in digests of benign human prostate tissue.

When each of these populations, along with urogenital sinus mesenchyme cells that promote the proliferation of primitive prostate cells, was injected subcutaneously into immunodeficient (NODSCID- IL2Rγ−/−) mice, the basal cell population gave rise to prostate-like structures containing both basal and luminal cells, whereas the luminal population did not grow, confirming observations from mouse prostate (4) that the basal layer contains prostatic epithelial stem cells.

Goldstein et al. (3) then used lentiviral vectors to transform these cells with genes encoding activated Akt and ERG, which are commonly associated with human prostate cancers. When transplanted into the mouse, the transformed basal cells formed tissues that resembled prostatic intraepithelial neoplasia (PIN) (that is, microscopic groups of atypical epithelial cells that represent a premalignant state), containing both basal and luminal cells, whereas transformed luminal cells did not grow.

Finally, addition of the androgen receptor gene, which is often up-regulated in prostate cancer, to the genes expressing activated Akt and ERG in the basal cells gave rise to frank adenocarcinomas with an expanded luminal cell population and an absence of basal cells, whereas expression of these same genes in luminal cells did not generate any prostatic tissue.

The authors conclude that basal stem cells are the target of transformation in the generation of prostate tumors.

Finally, in the recent study by Zhang et al they conclude:

The current study has made the following significant findings (see Supplementary Discussion).

First, our study uncovers unique SC- and EMT-enriched gene-expression profile in unperturbed basal cells that support the long-held hypothesis that the human prostate basal cell layer harbors primitive SCs.

Second, we report the surprising finding that basal cells are enriched in genes normally associated with neurogenesis. In contrast, luminal cells preferentially express proneural genes involved in neural signal response and processing. Consistently, primary basal cells can spontaneously or be induced to undergo 'neural' development in vitro, generating NSC-like cells. Combined with the SC features, these transcriptional programs provide a molecular understanding for the reported basal cell plasticity.

Third, basal cells express high levels of Pol I-associated rRNA biogenesis genes regulated, at least in part, by the MYC transcriptional programme. MYC is often found overexpressed in PCa, especially metastatic PCa. Increased transcription of rRNA genes by Pol I is a common feature of human cancer. Thus, our data may suggest a rationale for treating anaplastic PCa and CRPC *with Pol I inhibition, as well as targeting MYC and the MYC-mediated transcriptional programme as a therapy for PCa.*

Fourth, our deep RNA-Seq data provide a rich resource for epithelial lineage specific genes and markers in the human prostate.

Fifth, distinct transcriptomes in basal and luminal cells also suggest cross communications between the two epithelial cell types, as well as between the epithelial compartment and the underlying stroma. Understanding such crosstalk will be instrumental for understanding the normal development and tumorigenesis of prostate. Although many of the signaling pathways mentioned in this study are poorly investigated in normal prostate epithelial biology, their functional involvement in PCa development and progression has been widely documented3.

Last, the basal cell gene-expression profile is linked to adverse clinical features of PCa, indicating a 'biomarker' value of basal cell gene signature for aggressive PCa.

Importantly, the molecular resemblance of basal cells to anaplastic PCa and CRPC provides a common molecular understanding of these diverse and poorly characterized aggressive PCa subtypes and implicates basal cells as the cell-of-origin for these variant PCa. It should be noted that while this manuscript was under review, another paper reported similar findings in linking the basal cell gene expression to aggressive PCa

The above by Zhang et all appears to be the most comprehensive argument for CCO as basal.

There is a set of counter proposals for the luminal cells as the CCO.

Specifically, another set of arguments defends the luminal cell, namely Wang and Shen have noted:

Other studies have provided evidence that luminal cells can serve as cells of origin for prostate cancer. For example, pathological analysis of high-grade PIN samples, which still retain basal cells, suggest that molecular events associated with human prostate cancer initiation such as upregulation of c-MYC and shortening of telomere length occur exclusively in luminal cells but not their basal neighbors. In mouse models, a recent study using a prostate-specific antigen-Cre, PtenloxP/loxP prostate cancer model reported that the initial hyperplastic cells were all luminal. Finally, our laboratory has shown that targeted deletion of Pten in CARNs resulted in high-grade PIN and carcinoma, indicating that CARNs are a cell of origin. At present, however, it is unknown whether CARNs exist in the hormonally intact prostate epithelium, and if so, whether these cells can serve as cells of origin. Indeed, if CARNs correspond to facultative stem cells, as discussed above, they may correspond to a cell state that is only acquired in the regressed epithelium.

Also from Moscatelli and Wilson we have the arguments:

At first glance, these findings seem to be in conflict with those in a recent paper from Wang et al. (5) that concludes that a luminal epithelial stem cell is the target of transformation in prostate cancer. This conclusion relies on lineage-tracing studies in the mouse prostate. Wang et al. (5)

found that expression of a prostate-specific homeobox gene, Nkx3-1, marked rare luminal epithelial cells but was never observed in basal cells in prostates after castration-induced involution. When mice are castrated to abolish the production of testicular androgens, the prostate involutes, resulting in a reduction in size due to apoptosis of most luminal cells and of a small fraction of basal cells.

When androgens are readministered, the prostate regenerates. When castration-resistant Nkx3-1–expressing cells (CARNs) marked with yellow fluorescent protein (YFP) were followed, it was found that these cells expanded over ninefold during regeneration of the prostate after androgen replenishment and gave rise to luminal, basal, and neuroendocrine cells.

Reimplantation of single YFP-marked CARNs, along with urogenital sinus mesenchyme, under the renal capsule (a fibrous layer surrounding the kidney) of immunodeficient (nude) mice generated prostatic ducts containing both basal and luminal cells that were completely YFP positive.

Specifically deleting the tumor suppressor gene PTEN (which regulates the Akt signaling pathway and is often inactivated in human prostate cancer) in CARN cells led to the rapid development of tumors with a luminal phenotype and an absence of basal cells upon prostate regeneration. These results suggested that CARNs are prostate stem/progenitor cells and targets of transformation.

Similarly, in a recent (2015) paper by Agarwal et al the authors note:

Primary prostate cancer almost always has a luminal phenotype. However, little is known about the stem/ progenitor properties of transformed cells within tumors. Using the aggressive Pten/Tp53-null mouse model of prostate cancer, we show that two classes of luminal progenitors exist within a tumor. Not only did tumors contain previously described multipotent progenitors, but also a major population of committed luminal progenitors.

Luminal cells, sorted directly from tumors or grown as organoids, initiated tumors of adenocarcinoma or multilineage histological phenotypes, which is consistent with luminal and multipotent differentiation potentials, respectively.

Moreover, using organoids we show that the ability of luminal-committed progenitors to self-renew is a tumor-specific property, absent in benign luminal cells.

Finally, a significant fraction of luminal progenitors survived in vivo castration. In all, these data reveal two luminal tumor populations with different stem/progenitor cell capacities, providing insight into prostate cancer cells that initiate tumors and can influence treatment response.

Thus using this model, we again see an argument for luminal cells.

8.8.5 PI3K/AKT

The PI3K/AKT pathway is a significant pathway as we have shown. As Robbins and Hague note:

PI3K/Akt signaling in human cancer can be driven by tyrosine kinase receptors, G-coupled protein receptors, or mutant RAS. PI3K catalyzes the production of the lipid second messenger, phosphatidylinositol trisphosphate (PIP3) from phosphatidylinositol bisphosphate (PIP2). The action of PI3K is antagonized by PTEN, which dephosphorylates PIP3 at the 3′ position, returning it to its inactive form. Akt specifically binds the 3′-phosphorylated inositol lipids via its plekstrin homology domain, hence PIP3 recruits Akt to the cell membrane.

Akt–PIP3 binding results in a conformational change that opens up the C-terminal kinase domain of Akt for activation by phosphorylation. The Akt family comprises three highly homologous serine- threonine kinases: Akt1, Akt2, and Akt3. For activation, each isoform requires phosphorylation at equivalent threonine and serine residues in the Akt1/2/3 molecule: first, close to the active site at Thr308/309/305 in a region termed the activation loop mediated by phosphoinositide-dependent kinase 1 (PDK1); and second, in a C-terminal hydrophobic motif at Ser473/474/472.

The kinase responsible for the serine phosphorylation has been debated, and kinases that can phosphorylate at this site include integrin-linked kinase (ILK), protein kinase Cβ2, DNA-dependent protein kinase (DNA-PK), and ataxia telangiectasia mutated (ATM) (7). Akt can also autophosphorylate at this residue if the plekstrin homology domain is exposed (allowing recruitment to the plasma membrane) and the threonine residue (Thr308 in Akt1) is phosphorylated.

However, the mTORC2 complex is now believed to be the main kinase responsible for phosphorylation at the Ser473/474/472 position….

The role for the PI3K/Akt pathway in **thyroid carcinogenesis** *was first suggested by propensity of patients with Cowden's syndrome, an autosomal dominant multi-organ hamartoma syndrome, to develop thyroid tumors. Germline mutations in PTEN underlie 80% of Cowden's syndrome cases. Other cases result from germline promoter hypermethylation resulting in transcriptional down-regulation of KILLIN, a pro- apoptotic gene sharing PTEN promoter sequences, or germline mutations and variants in the succinate dehydrogenase genes (SDHx), which result in elevated Akt and MAPK signaling, or mutation in either PIK3CA or AKT1 …*

There is growing evidence from examination of Akt phosphorylation that Akt signaling has a role in sporadic thyroid cancers examined levels of p-Akt(Ser473) and Akt1, Akt2 and Akt3 in 8 follicular thyroid cancers, 9 papillary thyroid cancers, and 11 normal thyroid samples by western blotting and observed increased Akt1, Akt2 and p-Akt(Ser473) in follicular but not papillary cancers.

By contrast, also using western blotting, …compared seven matched pairs of papillary thyroid cancer and adjacent normal tissue and found total Akt levels unchanged, but there was significantly higher p-Akt in tumor tissue compared to surrounding tissues (five of seven pairs), with elevated levels of activated downstream targets p-Bad and p-p70S6K. … found elevated levels of p-Akt(Ser473) in six out of eight papillary thyroid cancers compared to adjacent normal *tissue, whereas total Akt levels were similar. ...used an ELISA method, and found enhanced levels of cytoplasmic Akt1 in 23 thyroid carcinomas compared with 16 non-neoplastic lesions (nodular goiters). The majority of Akt1 was cytosolic in differentiated cancers whereas two of three anaplastic cancers showed lower cytosolic and higher nuclear Akt1 expression.*

However, when Akt1 immuno-precipitates were examined by immunoblotting, the ratio of p-Akt(Ser473) to total Akt1 was lower in cancers compared to normal tissues (although only a subset of the tumors were shown to be analyzed in this way and it is unclear if the anaplastic cancers with high nuclear Akt1 were included).

On this basis, the authors proposed that either Akt2 or Akt3 might be activated in thyroid cancers rather than Akt1.

As we saw previously, PI3K and AKT play a significant up-path role in many of the driving pathways in a cell. These have also been considered as therapeutic targets but since they play a pervasive role in many cell activities, general targeting would be problematic. The Akt1 variant may be of some interest as noted above, yet the authors note that Akt2 and Akt3 are of more interest.

8.9 CONSIDERATIONS

The various sides of the arguments presented herein most likely continue. As much as murine models have value they also are a substantially different species.

8.9.1 How Close is Close?

The issue of how close we should be examining the tumors is a critical one. As Gundem et al have noted the PCa tumors are very genetically heterogeneous. In the development of a metastatic state the original tumor spreads and optimizes itself to the environment in which it is best suited. Thus as is frequently the case the PCa tumors seek presence in the bone and restructure the bone in their own liking. The question is then; what are the spatio temporal changes we see and can they become elements of therapeutic targets?

To understand this better, we again examine the literature. In the conclusion to the Navin and Hicks paper they state:

Biological models are by definition built upon incomplete information. At best, these explicit models for tumor progression provide guideposts for further exploration. As technology continues to evolve, the analysis of cancer samples of complex mixtures will give way to methods aimed at the individual cell.

Such methods will enable single cancer cells to be tracked as they progress to form the primary tumor and traced as they migrate through the body to seed the metastasis. In the near future the cost of deep sequencing a mammalian genome, whether from a tumor sample or a few disseminated cells will be approximately equivalent to the current price of a microarray experiment. Single cell genomes are also ideal for constructing detailed lineages of tumor *progression, because individual mutations in a genome can be traced as they are inherited and expanded in subpopulations.*

As we bring the magnifying glass closer, we may also be able to track the genetic stepping stones for tumor growth, or follow the genetic changes in circulating tumor cells as they progress from the primary to metastasis. Perhaps, we will find evidence that individual circulating tumor cells return to the primary tumor after developing offsite as the self-seeding model suggests. It is then that these predictive genetic models will have *realized their full value.*

It is reasonable to consider that examining the cell by cell profile of a cancer will be exceptionally enlightening. In addition to understand from the tumor progression how the malignancy changes in time and place is also critical. The issues as to what causes a cell to proliferate and mutate is essential to understanding how to target the cell. Perhaps if the CSC model is correct and that if we target the CSC itself then the other cells just die off.

8.9.2 Cell Import

What cell should we focus on and how do we identify it? As much as we have gathered about PCa and its genetics, we are still often in the dark because we lack the equivalent of the simplicity of a set of Newton's Laws. The state of a PCa cell is stochastic and does not follow the ballistic parabolic flight of a Newtonian projectile. Thus "Moon Shots" are problematic at best. We may still be hurling stones from Roman like launches.

From Agarwal et al:

This study characterizes primary prostate tumors initiated by loss of the common tumor suppressors, Pten and Tp53, for stem/progenitor phenotypes as assayed by in vitro organoid cultures and in vivo tumor-initiating activity.

It has not been routinely possible to culture luminal stem/progenitor cells, which has prevented ex vivo analysis of these important cells in primary prostate tumors, biasing most studies toward primary basal cells or human prostate cancer cell lines.

We have observed two classes of self-renewing luminal progenitors in Pten/Tp53-null tumors, a minor population giving rise to multilineage organoids (multipotent progenitors) and a major population producing luminal-only organoids (luminal committed progenitors). Of particular interest is the observation that multilineage organoids give rise to self-renewing luminal organoids, providing additional insight into progenitor subpopulations, lineage stages leading to luminal commitment, and one route of prostate adenocarcinoma mitogenesis.

We suggest that combined loss of Pten and Tp53 either in the luminal multipotent progenitor or a precursor has revealed a naturally transient population, possibly by inhibiting the normal rate of differentiation. This interpretation is consistent with considerable evidence linking Tp53 to the regulation of differentiation in stem cells.

To date, luminal multipotent progenitor cells have not been observed in lineage tracing experiments, except in the case of rare CARN's, prompting questions about the significance of *the multipotent progenitors revealed in organoid cultures. We show the existence of multipotent and luminal-committed TICs isolated directly from tumors, producing either adenosquamous carcinoma or adenocarcinoma, respectively. Importantly, the TIC assays used here measured autonomous differentiation potential in the absence of inductive embryonic urogenital mesenchyme. Endogenous adenosquamous prostate carcinoma is observed in a fraction of PB-CRE4; Ptenfl/fl;Tp53fl/fl mice, supporting the concept that transformed multipotent progenitors exist in vivo and can differentiate to both basal and luminal lineages in tumors in situ.*

It seems likely that the microenvironment will influence lineage commitment, and we note that organoids and TIC assays are performed in the absence of stromal cells. Therefore, it is possible in these assays that the extent of basal cell commitment by multilineage progenitors may be increased relative to the endogenous microenvironment.

Although engineered models of prostate cancer are often used to analyze the consequences of combined genetic mutations, the effect upon stem/progenitor populations has not been commonly considered. We show here for PB-CRE4-initiated genetic changes that Tp53 in combination with Pten loss demonstrated significantly different stem/ progenitor populations compared to Pten loss alone.

Specifically, Tp53 loss leads to the presence of luminal multipotent stem/progenitor cells and a self-renewing luminal population, correlated with accelerated adenocarcinoma development, that is absent in Pten-null prostates. In addition, it is possible that Tp53 loss primes for lineage plasticity, similarly to the phenotypic dedifferentiation of luminal mammary epithelium following Brca1 loss. Analyses of stem/progenitor populations contribute fundamental knowledge for molecular and pathological comparisons of GEM models and for interpretation of target populations responding to therapeutics…

Due to a lack of biomarkers, the extent of innate stem/progenitor subpopulation heterogeneity in human prostate cancer is not known.

The metaphor of launching stones is apropos. We cannot truly identify the targets and we do not have the predictive tools of Newton.

In a recent paper by Smith et al the authors have a model somewhat akin to what we presented several years before. Namely they state:

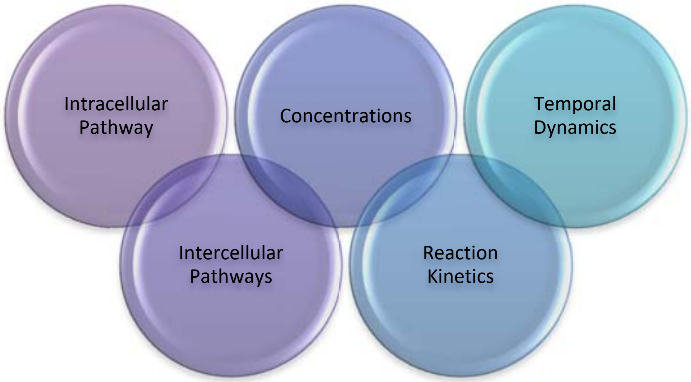
By analyzing stem cell differentiation dynamics in many spatially defined microenvironments, we found strong stochastic behavior during the differentiation process. The composition of individual micropatterns varied dramatically over the time course of the differentiation. On smaller micropatterns, we observe that the most probable composition is either 100% stem cells or 100% differentiated cells.

Moreover, the physical dimensions of the microenvironment can influence stem cell differentiation in significant ways. We propose a stochastic differentiation model frame-work, and showed that stem cell differentiation probability is a strong function of local stem cell fraction within the immediate cell vicinity.

When stem cells are surrounded by other stem cells, the differentiation decision is slow; whereas, when differentiated cells surround stem cells, then the differentiation rate is faster by nearly threefold. This result is consistent with the previous proposal that there are feedback signals between differentiated cells and stem cells16. The proposed stochastic modeling framework should be applicable in other settings for understanding differentiation dynamics. We also found that the cell-cell interaction during differentiation is partially mediated by an E-cadherin governed signaling mechanism. Although, cell-cell interaction is not completely inhibited in our experimental conditions, we are able to manipulate, observe, and quantify variances in differentiation kinetics when the roles of cell contact in spatially confined domains are altered.

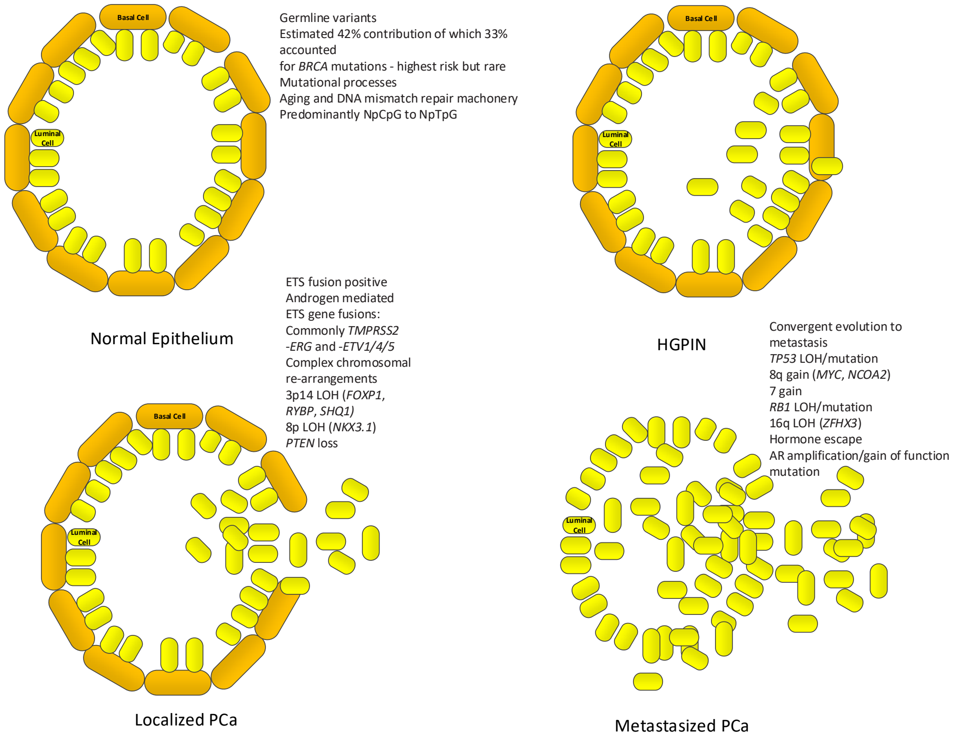
It is possible that E-cadherin affects multiple sensing mechanisms in stem cells and there are redundant mechanisms that reinforce cell-cell interaction in stem cell niches.

We have demonstrated a model containing the key elements shown below.



In a sense this is also what Smith et al are trying to develop. We believe that by examining the cancer in a large scale stochastic manner we can utilize current knowledge and develop new understanding. The cancer in our model is considered almost as a separate entity existing in a human body, and it uses the characteristics of its carrier, the human, to facilitate its growth. The human is in homeostasis and the cancer entity is competing with the human for resources to survive and prosper.

Considerable understanding on the details of PCa cell complexity has become available recently (see Gundem et al and Mitchell and Neal) From Mitchell and Neal we have the following Figure:



The question then is: in this phenomenological complex, what is the role of the CCO and CSC? One can consider the gene expression changes, due to mutations, epigenetic factors or otherwise, then combined with ligands that prompt pathways to operate and for the gene expression changed cell to proliferate and/or produce other growth factors and/or impact the extracellular matrix changing adhesion to see this new "organism", the tumor mass, to spread and alter itself to maximize its growth potential.

In essence we have a Darwinian sub-process allowing this new "organism" to prosper. To counter this process, we must identify the control mechanisms, all, not just a few, and then suppress them.

8.10 STEM CELLS AND THYROID CANCER

We can now address this issue in the context of thyroid cancers. As Guo et al have noted:

Thyroid cancer is one of the most rapidly increasing malignancies in the USA. Although most well-differentiated thyroid cancers have a good prognosis, a small percentage of these tumors are associated with recurrence and metastatic disease. Multidisciplinary treatment strategies are used for thyroid cancer, including surgical resection, targeted or cytotoxic chemotherapy, radiation therapy, or a combination of different treatments.

A small percentage of patients with well-differentiated thyroid cancers develop rapidly progressive disease, which is resistant to treatment. While patients with anaplastic thyroid carcinomas (ATC) usually succumb to their disease within a year after diagnosis regardless of the treatments used. One model that may explain the aggressive behavior of some thyroid *cancers, such as anaplastic cancer, the development of recurrent disease and metastases after surgery, and the resistance of some thyroid cancers to chemotherapy and radiation therapy involves the existence of cancer stem-like cells (CSCs).*

The CSC model (hierarchical model) postulates that a small population of cells in the cancer is responsible for tumor initiation, growth, and recurrence. Such cells are thought to exist in tumors as a distinct subpopulation and cause relapse and metastasis by giving rise to new tumors. Therefore, development of specific therapies targeting CSCs should improve survival and the quality of life of cancer patients with aggressive thyroid cancer. This model agrees with the fetal cell carcinogenesis of the thyroid, theory of Takano, which suggests that stem cells give rise directly to well differentiated and undifferentiated thyroid carcinomas

We have previously discussed the EMT process as an inherent element of metastasis. Guo at al note:

Epithelial tumor cells gain invasiveness and migratory abilities in the process of EMT, which is essential for successful metastatic spread. During EMT, epithelial cells lose their polarized organization and cell–cell junctions, undergo changes in shape to a mesenchymal or fibroblast-like cell morphology, and show increased cell migration and invasion. The process of EMT is likely responsible for decreases in drug efficiency and failure of some anticancer therapies. EMT is a multi-step process involving molecular and cellular changes in epithelial cells and in the adjacent stroma. Restrained and immobile epithelial cells gain a mesenchymal phenotype, characterized by enhanced motility and ability to degrade ECM.

This leads to decreased cell–cell adhesion due to the downregulation of epithelial proteins, mainly E-cadherin, but also claudins, occludins and cytokeratins. The cells that have undergone EMT show changes in apicobasal polarity, contributing to the spindle-shaped morphology. The characteristic features of a mesenchymal phenotype are high expression of N-cadherin as well as fibronectin (FN), vimentin, tenascin C, collagen VI-a, and laminin-b1. These changes observed in the EMT process are governed by transcription factors such as Twitst1, Snail, Slug, ZEB1, and ZEB2. To date a number of different EMT inducers such as Wnt, Hh, EGF, and TGFb have been described and some of the molecular pathways have been delineated.

Todoro et al have remarked:

Thyroid carcinoma is the most common endocrine malignancy and the first cause of death among endocrine cancers. We show that the tumorigenic capacity in thyroid cancer is confined in a small subpopulation of stem-like cells with high aldehyde dehydrogenase (ALDHhigh) activity and unlimited replication potential.

ALDHhigh cells can be expanded indefinitely in vitro as tumor spheres, which retain the tumorigenic potential upon delivery in immunocompromised mice. Orthotopic injection of minute numbers of thyroid cancer stem cells recapitulates the behavior of the parental tumor, including the aggressive metastatic features of undifferentiated thyroid carcinomas, which are sustained by constitutive activation of cMet and Akt in thyroid cancer stem cells. The identification of tumorigenic and metastagenic thyroid cancer cells may provide unprecedented preclinical tools for development and preclinical validation of novel targeted therapies….

The possibility of prospectively isolating and growing tumorigenic clones from thyroid cancer has considerable implications. An extensive characterization of tumor-initiating cells may allow the identification of new biomarkers for prognostic and therapeutic purposes.

Moreover, these cells can be screened for drug sensitivity or used for generating novel animal models of thyroid cancer. In this context, the orthotopic delivery of thyroid cancer stem cells is able to recapitulate the aggressive behavior of undifferentiated thyroid carcinomas, including local and distant metastases. Because anaplastic thyroid tumors are invariably lethal, this orthotopic model could be exploited for experimental testing and preclinical validation of new treatments.

Thus there is significant work and interest in isolating and identifying the characteristics of the thyroid stem cells.

8.11 CSC AND MIRNA

Now we have also noted a nexus between the CSC and miRNAs. On one hand the nexus was related to the flow of exosomes carrying miRNAs from the CSC to other cells both proximate to the CSC as well as distant from it. Perhaps we have conjectured this may play a significant role in metastasis.

As Liu and Tang have noted:

Cancer stem cells (CSC), or cancer cells with stem cell properties, have been reported in many human tumors and are thought to be responsible for tumor initiation, therapy resistance, progression, relapse, and metastasis. Despite their potential clinical importance, how CSCs are regulated at the molecular level is not well understood.

MicroRNAs (miRNA), small noncoding RNAs that play critical roles in normal stem cell functions during development, have emerged as important regulators of CSCs as well. In this review, we summarize the current major findings of miRNA regulation of various CSCs and discuss our recent findings that miR-34a suppresses prostate CSCs and metastasis by directly repressing CD44. This recent progress has important implications for understanding how CSCs are intricately regulated by networks of miRNAs and for developing novel mechanism-based miRNA therapeutics that specifically target CSCs.

9 IMMUNOTHERAPEUTICS

There has been a significant set of developments in the area of cancer immunotherapy over the past decade. Since much of the early work of Rosenberg and his colleagues, immunotherapy has grown by leaps and bounds as a hand in glove understanding of the system and of the therapeutics has evolved. We have discussed this at length elsewhere and will not delve into this here[58]. As Ward has noted:

The association between thyroid cancer and thyroid inflammation has been repeatedly reported and highly debated in the literature. In fact, both molecular and epidemiological data suggest that these diseases are closely related and this association reinforces that the immune system is important for thyroid cancer progression. Innate immunity is the first line of defensive response. Unlike innate immune responses, adaptive responses are highly specific to the particular antigen that induced them.

Both branches of the immune system may interact in antitumor immune response. Major effector cells of the immune system that directly target thyroid cancer cells include dendritic cells, macrophages, polymorphonuclear leukocytes, mast cells, and lymphocytes. A mixture of immune cells may infiltrate thyroid cancer microenvironment and the balance of protumor and antitumor activity of these cells may be associated with prognosis. Herein, we describe some evidences that immune response may be important for thyroid cancer progression and may help us identify more aggressive tumors, sparing the vast majority of patients from costly unnecessary invasive procedures. The future trend in thyroid cancer is an individualized therapy.

Ward also continues with a discussion of the Toll Like Receptors, TLRs, which play an interesting role. She notes:

Both a causal association and a noncausal association between thyroid cancer and CLT have been proposed. The molecular mechanism that links inflammation and cancer is not completely clear so far. A link between thyroid cancer, in particular the PTC histotype, and thyroid autoimmunity has long been recognized, although the precise relationship between the two diseases remains subject of debate. Herein, we list some evidences of molecular mechanisms that may be involved in both thyroid cancer and thyroid autoimmunity.

Pathways of immune activation could exert a role in thyroid cancer and CLT link. Toll-like receptor (TLR) comprises a family of cell surface receptors involved in the recognition of pathogen-associated signature molecules that signal the activation of innate and adaptive immunity. TLR family consists of more than ten members, and TLR3 had been reported to be restricted primarily to dendritic cells of the immune system …

TLR3 can be functionally overexpressed in cultured human thyrocytes by stimuli. Immunohistochemistry showed that TLR3 protein is overexpressed in human thyrocytes surrounded by immune cells in all patients diagnosed with Hashimoto's thyroiditis, suggesting

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

that TLR3 overexpression can induce an innate immune response in thyrocytes, which may be important in the pathogenesis of HT and in immune cell infiltrates …PTC cells basally express TLR3 RNA and that TLR3 signal systems are functional in these cells. High basal TLR3 levels and TLR3 signals capable of increasing cytokines and chemokines in PTC cells in vitro are consistent with the existence of immune cell infiltrates in vivo, based on related studies suggesting that elevated TLR3/TLR3 signals in HT are associated with immune cell infiltrates

TLRs are of interest. The key factor is inflammation. It is known that many thyroid malignancies appear to result from prior inflammatory states. In fact the anaplastic malignancy generally has a Graves like initiation.

9.1 SURFACE MARKERS

Surface markers are often seen in many solid cancers. Moeller and Fuhrer has presented details on thyroid receptors and cancers. It may be possible to exploit these markets as a means to detect and then attack the malignant cells with immunotherapeutic methods. The authors have concluded:

Epidemiological data suggest a tumor-promoting effect of TH as the incidence of some tumors (colon, breast, prostate, and lung cancer) was found to be increased with increasing TH whereas other tumors, e.g. breast cancer, occur later and are diagnosed in a less advanced stage in patients with hypothyroidism. In tumor transplant rodent models, TH appear to stimulate tumor growth and metastasis, whereas hypothyroidism has opposite effects.

Therefore, the results of clinical studies showing that treatment-induced hypothyroidism is associated with a favorable outcome in several cancer types, most prominently in RCC, are immediately comprehensible. In two prospective studies of RCC treated with sunitinib, development of hypothyroidism was an independent predictor of treatment success. Furthermore, in the only study replacing T4 in every RCC patient with elevated TSH and including only patients with normal TSH in the final analysis, no difference in treatment outcome between the patients that developed hypothyroidism and those that remained euthyroid was observed.

It is tempting to assume that induction of hypothyroidism is one of the mechanisms through which TKIs slow tumor growth and that correction of hypothyroidism eliminates the survival advantage. There is reasonable concern that substituting these cancer patients with T4 could deprive them of the potential beneficial effects of hypothyroidism.

Yet, one must be cautious because it cannot be ruled out that hypothyroidism is only a surrogate marker of antitumor treatment efficacy due to higher drug levels or different drug metabolism or susceptibility of the immune system in a subset of patients and does not influence tumor growth on its own. There may also be a bias because cancer patients on successful, and therefore continued, treatment are exposed to higher cumulative doses and have more time to develop hypothyroidism than rapidly deteriorating patients.

Additionally, a tumor-promoting effect of TH may only be found in certain types of cancer, such as glioblastoma and RCC, but not for others, e.g. HCC. Given the results of the tumor implant

animal models and the TKI trials in cancer patients, it is of great clinical importance to determine whether and in which types of cancer hypothyroidism can contribute to prolonged survival and should be tolerated or, more provocative, should even be induced. To address these questions, future cancer treatment studies, especially with substances that can induce hypothyroidism, should be designed in a way that allows for an analysis of thyroid function status and its contribution on treatment outcome.
It is tempting to address this issue of the TH as a target but clearly this is still too early.

There is also a great deal of work in using PD-1 and PD-L1 as a means to excite and effect T cell attack on tumors. PD-1 is the surface receptor that can inhibit the effects of T cells on malignant cells. PD-1 blockers then allow the T cell to function and we have seen great success in this area. Bai et al have studied this in thyroid tumors and they have noted[59]:

PTC is the most common malignancy of the endocrine organs, and its incidence has increased in the past decade. Although PTC overall has a good prognosis, more than 10% of patients with PTC develop recurrence or distant metastasis after surgery, and some of these patients with succumb to PTC.

Although the molecular mechanisms responsible for the initiation and progression of PTC are not fully understood, the mitogen-activated protein kinase (MAPK) pathway has been implicated in the development of more than 70% of PTCs, as well as BRAF and RAS mutations and gene fusions involving the RET and NTRK1 tyrosine kinase. The BRAF mutations play an important role in this process, and the BRAF V600E is the most common alteration. In recent years, a novel mutation-specific antibody targeting BRAF V600E protein has become commercially available. Its specificity and sensitivity to identify the BRAF V600E gene mutation have been verified in many series of tumors, including melanoma and PTC, and it could be used as a surrogate of genetic detection of BRAF V600E mutation…

Our previous study indicated that PTC patients with psammoma bodies, compared to those without, had poorer disease-free survival. The significant correlation between PD-L1 expression and absence of psammoma bodies may indicate that PD-L1 expression is possibly associated with a favorable clinical outcome….

We examined the expression of PD-L1, PD-1, and BRAF V600E in 126 cases of PTC, and found that distinctive pathological features in PTC, including TILs, a background of CLT, psammoma bodies, and stromal calcification, were useful parameters for predicting PD-L1 or PD-1 expression, which was a novel finding and should be highlighted in the forthcoming era of immunotherapy. Further studies are necessary to determine the prognostic and therapeutic value of these findings in PTCs.

Overall a recent effort by Na and Choi examines papillary thyroid cancer and they note:

Although papillary thyroid cancer (PTC) is curable with excellent survival rate, patients with dedifferentiated PTC suffer the recurrence or death. As cancer immune escape plays a critical

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[59] See the paper by Chowdhury et al as well.

role in cancer progression, we aimed to investigate the relationship between differentiation and immune landscape of PTC and its implications for immunotherapy.

Using The Cancer Genome Atlas data, we estimated the immune cell enrichment scores and overall immune infiltration, ImmuneScore, to characterize the immune landscape of PTC. Thyroid differentiation score (TDS) was calculated from 16 thyroid function genes. We demonstrated that ImmuneScore had a significant negative correlation with TDS, and BRAFV600E+ tumors showed significantly low TDS and high ImmuneScore.

Enrichment scores of myeloid cells and B-cells were negatively correlated with TDS, while those of plasma cells were positively correlated with TDS. In addition, the association between TDS, ImmuneScore and immunosuppressive markers (CTLA-4, PD-L1, HLA-G) were evaluated according to BRAFV600E status.

All immunosuppressive markers expression had a significant negative correlation with TDS, and they were significantly higher in BRAFV600E+ status. Subgroups were divided by median values of TDS and ImmuneScore, and immunosuppressive markers of these subgroups were compared. The immunosuppressive markers expression was the highest in high ImmuneScore and low TDS subgroup. Furthermore, ImmuneScore had a significant association with recurrence-free survival, irrespective of clinicopathologic factors including BRAFV600E status.

These findings based on gene expression data illuminate the immune landscape of PTC and its association with TDS, immunosuppressive markers and recurrence. Our results would be extended to investigate immunotherapeutic approaches in PTC.

There is a challenge to find unique cell surface markers for a variety of cancers. Thyroid cancers may offer an interesting opportunity because of their location but they also tend to be highly heterogenic. That seems to be the major challenge.

9.2 INNATE VS ADAPTIVE METHODS

The innate and adaptive immune systems are the key players in attacking many cancers especially as they have been adapted to do so. In previous works we have examined both the innate and the adaptive systems. The innate system has a somewhat sledgehammer approach to what could be perceived as an unacceptable cell but recent efforts look favorably upon it. We examine three elements.

9.2.1 NK Cells

Natural Killer cells are part of the innate immune system. They have a strong propensity to attack and kill cancer cells but not on a systematic basis. In a recent work by Zhu et al they have studied this effect in some detail on murine thyroid cancer models. They have noted[60]:

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[60] See the paper by Mold et al as well for detailed remarks on NK cell capability.

Although differentiated thyroid cancer constitutes >90% of thyroid cancer cases and has an excellent prognosis, anaplastic thyroid cancer (ATC), an undifferentiated subtype of thyroid cancer, is one of the most fatal malignancies, with a mean survival of 2–6 months and an overall 5-year survival rate of up to 14%.

The poor prognosis of ATC is known to be partly due to early metastases of the malignancy, which are not surgically removable and are unresponsive to conventional chemotherapy or radiotherapy. Pulmonary metastasis of ATC is usually multifocal; therefore, these metastases are almost always not surgically resectable. New effective therapeutic strategies for pulmonary metastasis of ATC might improve the prognosis of this cancer. The immune system plays a crucial role in combating tumors, and it has promising potential in the oncologic arena.

Natural killer (NK) cells are able to kill target cells without prior stimulation, and their use is more feasible compared to T cells, which need prior sensitization.

The in vivo tumoricidal effect of NK cells on various malignancies, including thyroid cancer, has been reported in previous studies. ... human ATC cells are sensitive to NK cell- mediated lysis via ULBP2/5/6, and are able to chemoattract NK cells. The cytotoxic mechanism of NK-92MI in ATC has yet to be clearly explored. However, ... the mechanism is dependent on the expression level of NKG2D ligand on target cancer cells.

Furthermore, ... NK cells can inhibit pulmonary metastasis formation after IFN-γ treatment in a mouse model of colon cancer. The lungs are the most common metastatic site of ATC, followed by bone, and the metastases usually are not surgically resectable. Therefore, new effective therapeutic strategies for pulmonary metastasis of ATC are urgently needed, and NK cell- based immunotherapy might represent a therapeutic strategy for the metastases. The present study determined whether ATC pulmonary metastases would be a suitable target for NK cell- based immunotherapy.

9.2.2 TIL

TILs, tumor infiltrating lymphocytes are another class of natural tumor fighters. Uppaluri et al have noted:

The expanding and established literature that correlates tumor infiltrating lymphocytes (TILs) with outcomes of patients with solid tumors has contributed greatly to the appreciation of the interaction between the host immune system with neoplastic growth. This analysis has been limited to specific tumors, such as melanoma and ovarian cancer, and our understanding of TILs in relation to many other malignancies has yet to be explored. We review one less well studied malignancy, head and neck squamous cell carcinoma (HNSCC), and the initial attempts to examine the impact of TILs on outcomes of these patients.

To provide a context for the discussion of TILs and HNSCC, we first review the epidemiology, relevant head and neck anatomy, immune responses and discuss the historical data regarding the unique immunobiology of these tumors. Finally, with this perspective, we describe our current understanding of tumor infiltrating lymphocyte data for head and neck cancers.

Martins et al have noted specifically for thyroid cancers the presence and possible significance of TILs.

9.2.3 CAR-T

CAR-T cells are specifically engineered T cells that will attack tumors based upon specific surface markers on the tumor surface. They are quite successful in a multiplicity of cancers and in a recent paper by Martinez and Moon they reflect upon work being done in thyroid cancers. They note:

An in vivo study found that CAR T cells targeting ICAM-1, a marker associated with many solid tumors including thyroid cancer (but also expressed on many normal tissues as an adhesion marker), was safer and more effective when bearing CARs with micromolar affinity than with those with higher, nanomolar affinity[61] *. Additionally, the authors found that the CAR with lower affinity showed less exhaustion and enhanced proliferation in vivo.*

In another approach to limiting CAR toxicity, one group interested in treating colorectal cancer created a CAR targeting GUCY2C, a receptor that is conserved in at least 95% of metastatic tumor at tenfold greater levels, but is not targeted by T cells when expressed in normal epithelial tissues because it is restricted to luminal membranes (33). The CAR was shown to be safe and effective in both immunocompetent mice with metastatic tumors and human xenograft models. Antigens that are aberrantly or overexpressed on tumors but are also expressed on normal tissues…

It seems clear that the CAR-T cell approach has great potential yet they require an ability to target unique markers on the cell as well as being able to get to where the cell is. This may be a bit contradictory since most cancers, especially metastatic one have high vascularization and one would suspect that they would be readily targetable. This has yet to be proven in large scale trials.

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[61] Min I, Shevlin E, Vedvyas Y, Zaman M,Wyrwas B, Scognamiglio T, et al. CAR T therapy targeting ICAM-1 eliminates advanced human thyroid tumors. Clin Cancer Res. (2017) 23:7569–83. doi: 10.1158/1078-0432.CCR-17-2008

10 OBSERVATIONS

We can now make some overall observations. This is an attempt to draw together the multiplicity of issue we have discussed. It is important to understand that there are still a significant number of unanswered questions. However it is reasonable to assert that many if not most of the key questions may now be posed.

10.1 CANCER STEM CELLS

The cancer stem cell issue we have discussed is of prime importance. Consider what is currently done. If some palpable lesion is determined than an ultrasound is performed. The US may identify an area of interest. The an fine needle aspiration is performed. This is akin to say a prostate biopsy. It sample an extremely small section of the organ and the sampling is inclusive of but a few cells and is devoid of the underlying cell structure. One looks at the nucleus, the cell form, and perhaps a few other characteristics. Then the cells samples may get a NGS performed to ascertain what specific characteristics are present in that batch. At that point a decision is made on a partial or full thyrdoidectomy. It is only then that a more detailed identification histologically can be made.

However in no case have we identified a CSC, if such were present. Is the lesion removed one that contains the CSC or is it a satellite. It would be useful to have a system to sample on a cell by cell basis and to perform a HGS on each cell and to compare not only target genes but perhaps the whole genome; exons and introns. Moreover it may be useful to have an epigenetic mapping to see which parts of the DNA are methylated. Currently there are limited means to assess the histone epigenetic state.

Ideally this process could be accomplished and this of course would be a massive amount of data, yet using many of the data tools currently available one could examine the cells in toto. Perhaps identifying a CSC could result from this process.

Also it would be of interest to attempt to correlate the genetic and phenotypic characteristics of the cells, to be capable of improving histological analysis.

10.2 EPIGENETIC FACTORS

As we noted above, there is a need for a detailed mapping of epigenetic factors. Here is included not just methylation and possibly acetylation but a mass amount of miRNA assessment as well as lncRNA, siRNA and other fragments which could have an influence on gene expression. Part of the question could also be that some of the miRNAs are the result of mutation or other changes that give rise to them. The question would ne; what caused the change?

10.3 METASTASIS, EXOSOMES AND MIRNA

We have examined metastatic process at length elsewhere[62]. We know that certain miRNAs are present in thyroid cancers and that they play a significant role. The question should be; is it useful to assess tumors with their miRNA as well as fusions and mutations of genes? Furthermore the cells emit exosomes containing miRNAs and thus we should determine what they are. If they are one that can spread the malignancy, then perhaps this is a multistep process wherein distant cells get primed for the impact of other miRNAs at a later time. If the neoplasia is removed before such an event starts then perhaps a total cure is possible.

We do not fully understand the complex nature of the miRNA complex. One may start and others may follow but that is yet to be determined. At best we know of their presence and some of their functions. But as a system we seem to have little understanding.

10.4 IMMUNOTHERAPY

Immunotherapy is most likely the basis of the next frontier in cancer treatment. Patient heal thyself may be more than just a slogan, it may be a drive to understand, manipulate, and control the immune system. There are many ways to address this issue. They range from receptor matching and activation such as the use of PD-1 to the CAR-T cell approach where very targeted tumor cell surface targets are identified and T cells produced to attack them. We have also seen the use of NK cells used in the CIK, cytokine induced killer cells, against hematopoetic cancers. For thyroid cancers this is a fertile field to examine.

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[62] https://www.researchgate.net/publication/331318495_Exosomes_and_Cancer and https://www.researchgate.net/publication/330222973_EMT_and_Cancers

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