Exosomes and Cancer

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Exosomes are small walled vesicles that are emitted by a variety of cells. Malignant cells emit these many of which contain miRNAs. We examine a new paradigm for metastasis which incorporates this construct. This may lead to a novel set of therapeutics.

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Contents

1 INTRODUCTION

Exosomes and small vesicles, less than 100 nm, released by cells and containing parts of DNA or RNA, miRNAs, proteins, and other matter released by the cell. They become free in the extracellular areas and can find their way to other cell sites. The exosomes with miRNA may find other cells, attach, enter, and activate the cell in such a manner to result in the damage in the originating cell.

This paper is a combination of fact and speculation. It attempts to refer to many significant works presented in the literature while attempting to knit together an alternative view of metastasis. There is a great deal of evidence for this new paradigm, but the reader should be warned that there is no definitive acceptance. Thus the speculation. Paradigm shifts are always difficult. Kuhn and his followers have posited ways in which this occurs. I am hardly suggesting that there is any sudden or great insight, I am merely suggesting an alternative view. This view looks at the exosome and miRNA.

One could look at this as an example of a cancer cell of origin and metastatic cells at distant points. In this paradigm the cancer stem cell, "cell of origin", just sends out exosomes of miRNA which somehow float about until the find a cell to attach to. If one accepts this paradigm, it changes in material ways how we see metastasis and more importantly how we see possible therapeutics. Namely if a melanoma mets to the lung, does it do so via an miRNA in an exosome and moreover is it the lung because the lung tissue has a receptor that allows the entry of the miRNA.

Thus exosomes with miRNA can be powerful transmitters of cancers. The actual malignant cell does not have to move, it just has to send out the right miRNA.

As Rak noted regarding the work of Leyden and his Lab:

The metastatic dissemination of cancer cells from their site of origin through the bloodstream to distant organs is a major cause of cancer-related deaths. This process is not random1; instead, certain populations of cancer cells preferentially seek out and colonize specific organs, under the control of a range of molecular programs. Such homing implicitly involves interactions between cancer cells that escape the primary tumour, sometimes known as seeds, and the microenvironment, or 'soil', of target sites.

But less intuitive is the discovery by Hoshino et al. that seeds can influence the soil before their arrival, sending out extracellular vesicles called exosomes that precondition specific organs for metastatic invasion. There is growing support for the provocative notion that a build-up of systemic responses to a primary tumour might precede, and even enable, the eruption of metastatic cancer.

These responses might involve complex alterations in the body's vascular, coagulation and inflammatory systems — for example, cancer-related changes in the composition of soluble proteins, in cell populations or in the characteristics of exosomes5in the blood. Hoshino et al. define exosomes as small extracellular vesicles — membrane-bounded compartments that

transport proteins, lipids and nucleic acids from one cell to another, and which can travel considerable distances in bodily fluids or the bloodstream.

This information- transfer process has attracted considerable interest in cancer research, because some extracellular vesicles carry cancer causing genes called oncogenes, or oncogenic proteins that promote cancer formation and disease progression. The involvement of extracellular vesicles, including exosomes, in metastasis has been studied for some time, and contributes to several key events that prepare a distant site for colonization — a process called premetastatic niche formation.

Simply stated, it could be postulated that it is the exosome that initiates and facilitates metastatic growth, not necessarily the flow of the cells to new locations.

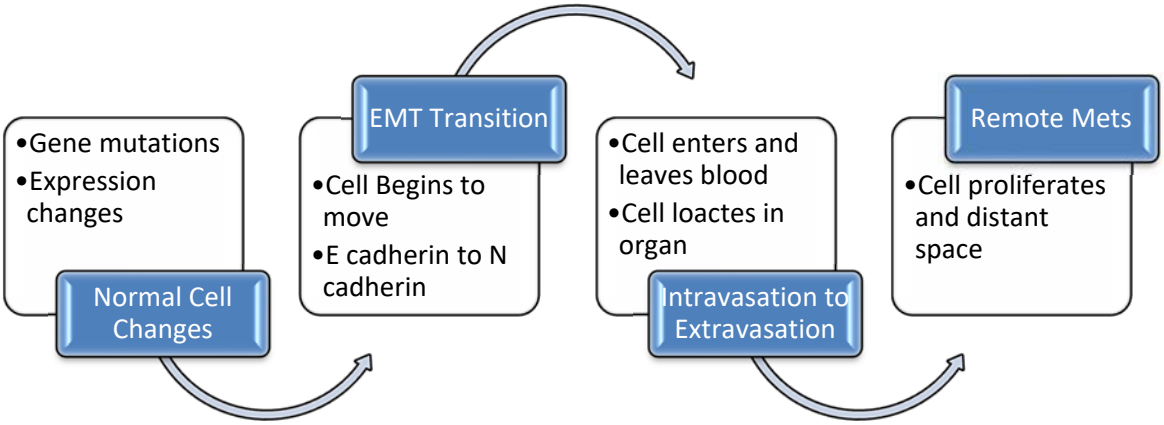
A great deal of effort is underway to resolve the extent of the functions of EVs in cancer metastasis and in turn the possibility of targeting them as a therapeutic.

There are two paradigms that we now work with in metastasis. The classic involves the movement of the malignant cell across the body. The second, the EV model, is the movement of EVs across the body, influencing distant phenotypes. The EV model makes sense in many cancers, because of the ease of the EV going into and out of the circulatory systems; blood and lymph.

We demonstrate these two paradigms below:

1.1 CLASSIC PARADIGM

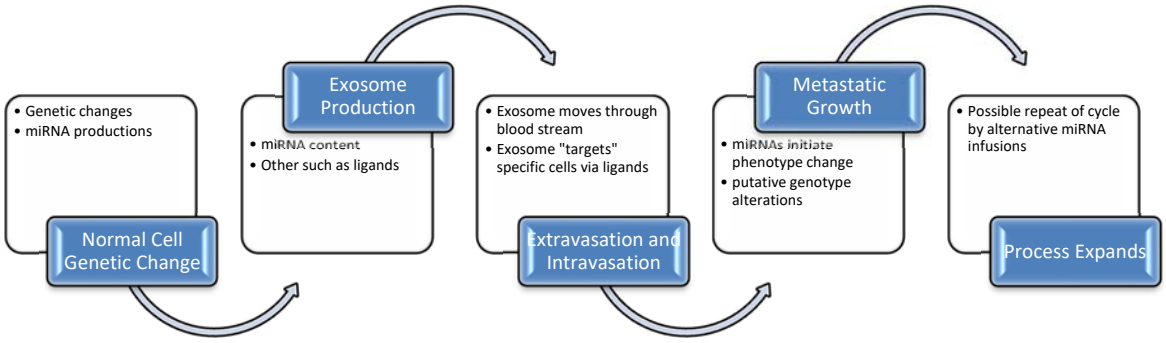
The classic paradigm is shown below. It fundamentally assumes that a single cell mutates and becomes malignant and then that cell proliferates as a cell and enters and leaves the blood system and finds a location where it can again proliferate, perhaps undergoing additional genetic changes. The key here in the classic paradigm is that the malignant cell is the mechanism for proliferation. The literature here is significant.



Key to this Classic Paradigm, is the belief that it is the malignant cell itself which migrates, and even more so, the cell is a stem cell. Namely it can migrate and do so in a manner that allows it to establish its own outpost of a malignancy. The cell maintains and carries with it the identical genetic flaws which made it what it is at its site of origin. Then as part of this paradigm, it is this cell which must go into and out of the blood stream. This behavior of the Classic Paradigm is a complex and oftentimes highly questionable type of behavior.

1.2 EV OR NEO-CLASSICAL PARADIGM

The new EV paradigm is shown below. It is not the actual cell but elements of the cell such as miRNA which go forth and multiplies. Note it first does a phenotype change still requiring fueling and then may actually have a genotype change resulting in the classic "whack a mole" results in metastasis.



The essence of the Neo Classical paradigm conjecture is severalfold:

1. A malignant cell is changed and produces exosomes which contain, for want of a better alternative, miRNAs which if and when absorbed by target cells are oncogenetic in nature. The malignant cell can best be called a stem cell.

2. The target cells absorbing and activating the miRNAs phenotypically become malignant. Their genetic structure has not changed but they are responding to the miRNA exosome. The target cells may be local or distant.

3. The target cells are targeted by ligands or receptors on the exosome that match those of the target cells. Thus there is a specificity of targeting, much like what we see in many metastatic events.

4. The exosomes are small enough and unencumbered with surface proteins that they are easily sent into and out of the blood stream. In addition they are almost invisible to the immune system.

5. Long term exposure is inducive to a genotypic change of the target cell, thus enabling it to become its own stem like cell.

6. Properly characterize exosomes may be targetable.

7. Early extraction of the stem cell before genotypic change can be curative. Once the target cell genotypically changes that cell must be excised or deactivated.

8. However, if the exosome itself is identifiable then perhaps an immune targeting may be achieved.

If the above conjecture, which we argue may have some validity, is correct, it argues for a dramatically different approach to cancer treatment.

1.3 ISSUES

Exosomes have become a significant factor in examining cancer metastasis. One could argue that they are paradigm shifting. Our focus herein is to examine exosomes as a significant if not primary diver of many metastases. This is a dramatically different view of cancer progression. It does allow for the explanation of many of the issue that we see in examining such progression.

1.3.1 What are exosomes

We use the literature to examine the exosomes and its kindred spirits. Exosomes are fundamentally ad double wall lipid carriers of cellular elements, predominantly parts of DNA, RNA, proteins, and most importantly miRNA.

1.3.2 What are the theories of metastasis

What is metastasis and how does it work. Again in a simplified sense, metastasis if the propagation of malignant behavior to other sites in addition to the primary site. Now trees and other plants get tumors. But these tumors do not spread. Large galls on the sides of trees are compartmentalized tumors plus viral materials. Thus what makes animals have such diseases and how do they function?

1.3.3 What are miRNAs and how do they relate to Exosomes

Micro RNAs are small RNA strips, about 22 base pairs in length, that can target and suppress mRNA inhibiting translation.

1.3.4 What is the impact of epigenetic factors on Exosomes

Epigenetic factors such as the impact of miRNAs can be significant. They silence or activate genes, they can also impact histones which in turn may silence or activate genes. We examine both to a degree. There is a complex network of positive feedback where miRNAs can induce via other genes favorable growth environments.

1.3.5 How do we understand the dynamics of cancers using the Neo Classical Model

In the Classical model we have the mutation of genes, the creation of a putative cancer stem cell, and the movement from one place to another via initially an EMT and then intra and extra-vasation. Now the Neo-Classical model assumes that it is the exosome and its contents, putatively the miRNA, that moves about. This can be a fundamental paradigm shift but arguably verifiable. Thus how then does one consider the dynamics of metastasis in this case?

1.3.6 What is the impact on therapeutics

Dealing with the exosomes one considers ways to block them via multiple therapeutics. If one can block them after initial tumor recognition then is there a way to mitigate metastasis?

1.3.7 How does Immunotherapy get applied

Immunotherapy has made great strides however to be effective the cells targeted must be recognizable. Thus understanding the surface of the exosomes or even more so to be able to target them and insert markers may be effective. One wonders if CAR-T approaches may be effective.

1.3.8 What can we do with diagnostics and prognostics

This is a key question which may be easier to deal with. We can now access many of the exosomes, examine their contents and then assess the specific malignancy and ascertain its progression.

We proceed to review, examine, and consider these issues. This is not a fundamental research paper since it relies on the work of others, it is not a review paper since it does not present a summary of others, but it is an attempt to consider a new paradigm. This may or may not prove to have sustainable capacity but the author believes it is worth the consideration in light of recent investigations.

2 EXOSOMES

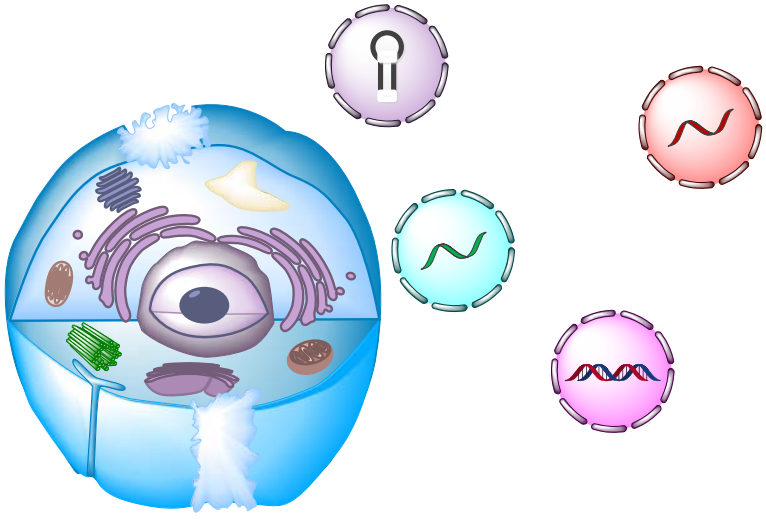
Exosomes are small vesicles that contain DNA fragments, RNA fragments, miRNA, proteins and other materials ejected from a cell. We give an graphic below. They are small, 10-90 nm in diameter, they are cell like with an shell and material inside, and somehow they can be directed, namely they may have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

2.1 TYPES

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[1].

	Exosomes	Microvesicles	Apoptotic bodies	Oncosomes
	Released by both normal and diseased cells	Released by normal cells	Released by apoptotic cells	Released by malignant cells
Size	40 - 120 nm	100 nm - 1 µm	50 nm - 2 µm	1 - 10 µm

The Figure below depicts some of the expelled cargo; miRNA, RNA fragments, DNA fragments, proteins.



[1] See Vader et al as well. They have a Table with slightly differing numbers. The above numbers are from the Breakefield Lab papers. (See Zaborowski et al) Also note (See Milo and Phillips) that a mammalian cell is typically 20 µm in diameter and has a volume of 3000 μm^3 That means that an exosome may be $1/1000^{th}$ of a cell.

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.

As Maas et al have noted regarding the specific structural issues:

EVs are thought to be formed by multiple mechanisms. In all cases, lipid curvature must be induced to form either an inward-budding vesicle within the endocytic system (exosomes) or an outward budding vesicle at the plasma membrane (microvesicles). For exosomes, several mechanisms have been described. The best-characterized mechanism involves recruitment of the endosomal sorting complex required for transport (ESCRT) machinery to ubiquitinated proteins in the early endosome. ...

A number of EV subtypes have been characterized. Traditionally, exosomes are small EVs (sEVs; < 150 nm) released through multivesicular bodies (MVBs) in the endosomal pathway. Vesicles can also bud off the plasma membrane, apparently in a manner similar to that of retroviruses, forming EVs in the 200-500 nm range. These shed vesicles are called microvesicles or ectosomes. However, smaller vesicles (~100 nm) have also been described to bud from the plasma membrane and may be isolated together with exosomes. Other modes of release include formation of EVs at the ends of microvillar-like protrusions, which can be accentuated by increased cellular content of hyaluronan In cancer cells, even larger EVs (1-10 pm in diameter), termed large oncosomes, can bleb off the cell membrane.

In addition, when cells undergo apoptosis they dissociate into membrane bound apoptotic bodies of different sizes, which are hard to distinguish from other types of EVs, but may contain relatively more genomic DNA. Due to the often unclear composition of purified vesicle preparations, which are usually isolated based on size and density, the terms sEVs and large EVs (lEVs) have been proposed for studies that do not clearly define the biogenesis mode of the EVs in their preparations

Now Zaborowski et al note further details regarding the structure. In fact as they note, the exosome is cell like with a lipid bilayer.:

EV membranes consist of a lipid bilayer similar to that of cell plasma membrane, in contrast to the single-layered high- and low-density lipoprotein (HDL and LDL) found in body fluids. Exosomes are enriched in sphingomyelin, gangliosides, and disaturated lipids, and their phosphatidylcholine and diacylglycerol proportion are decreased relative to the membranes of their cells of origin. Some studies also describe an increased fraction of cholesterol in exosomes compared with that in cellular membranes.

In contrast to cellular membranes, exosomes contain more phosphatidylserine in the outer leaflet, which may facilitate their internalization by recipient cells. A comparison of banked red blood cells and MVs derived from them revealed a high similarity in lipid composition, with the

exception of polyunsaturated glycerophosphoserine, which was enriched in MVs. These differences are consistent with the distinctive biogenesis of exosomes and MVs, because the latter stem directly from the plasma membrane.

2.2 STRUCTURE

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.

2.3 MOBILITY

Movement and interaction of the EV is a critical area of investigation. To function they must get somewhere. As Sullivan et al note:

The complex interactions displayed between cancer cells and the TME, as mentioned above, occur through a very complicated network of cellular communication. Many of these signaling pathways operate through direct cell-to-cell contact or using classical paracrine signaling loops of cytokines or growth factors with their receptors. However, more recently, EV shedding has

emerged as another important mechanism of cellular cross-talk. Extracellular vesicles are lipid bilayer-bound vehicles that are released from the cell membrane and carry nucleic acids (DNA, mRNA, and miRNA), proteins, and lipids to neighboring or distant cells.

Although EVs were first described over 30 years ago as being released from reticulocytes, they have gained significant attention only recently as key factors in regulating both normal cell physiology and disease states. They now have been identified in nearly all eukaryotic cells and prokaryotic cells and have been isolated from most bodily fluids. ...

EVs are classified into two groups depending on their size, biogenesis, and method of release from the cell. Exosomes are 30–100 nm in diameter and are generated within large intracellular multivesicular bodies.

They are released into the extracellular environment upon fusion with the plasma membrane. Microvesicles (MVs) generally range from 100 to 1,000 nm and are formed when cell components travel to the plasma membrane to be released by membrane budding. Due to an incomplete understanding of exosome and MV biogenesis, and inconsistent methods of purification, the two terms are sometimes used interchangeably within the literature. The classical protocols for purification such as ultracentrifugation, density gradient centrifugation, and newer commercially available kits have been shown to co-isolate MVs and exosomes as well as protein aggregates and other non-EV biomolecules that may interfere with EV specificity.

Since current isolation methods are not yet standardized, it becomes difficult to assign specific functions to exosomes or MVs independently and why they are both included under the broad classification of EVs.

The authors continue:

In the 1800s, Paget noticed that different tumor types tend to metastasize to specific organs leading to the "seed and soil" hypothesis of cancer metastasis. It is now well established that primary tumors can release cytokines, chemokines, and their receptors to direct metastatic cells to a preferred secondary site called the PMN. More recently discovered is that communication between the primary tumor and the PMN can be mediated through EVs.

For example, a repertoire of integrins have been reported to guide the vesicles to specific organs. EVs expressing integrin alpha-V beta-5 specifically bind to Kupffer cells mediating liver metastasis, whereas integrin alpha-6 beta-4 and integrin alpha-6 beta-1 bind lung-resident fibroblasts and epithelial cells to mediate lung metastasis.

The following we will come back to again and again. In this case the authors use an analog of a neutrophil possibly. This case is for a pancreatic cancer.

Once EVs arrive at the predetermined distant site, their cargo is unloaded to aid in a stepwise creation of the PMN. For example, pancreatic cancer cell EVs were shown to travel to Kupffer cells in the liver to deliver macrophage migration inhibitory factor. This induced the secretion of TGF-β in Kupffer cells and ultimately induced the recruitment of bone marrow-derived cells to complete PMN formation. Another important aspect of PMN formation is vascular leakiness,

which facilitates the extravasation of malignant cells via the delivery of specific molecules that trigger vessel permeabilization of endothelial cells including those carried in EVs. In one study, human breast cancer-derived EVs promoted vascular leakiness in the lung by upregulating S100 proteins and activating Src kinase signaling.

PMN or neutrophils have a tendency to aggregate around certain tumors. The question here is; are exosomes similar in collecting them as well?

Mathieu et al in a recent paper discuss the secretion dynamics of exosomes. They have noted:

…crosstalk between the intracellular molecular machineries involved in the biogenesis and secretion of EVs forming at the plasma membrane (microvesicles, microparticles or ectosomes) or in late endosomal MVBs (exosomes). Rab27a/b are involved in MVB-dependent secretion of exosomes but also in the release of viruses and Golgi-derived secretory granules. Ceramide generated by SMAses and the ESCRT machinery promote vesicle formation both at the plasma membrane and inside MVBs.

ARF6 and depolymerization of the actin cytoskeleton are required for EV secretion at both locations. By contrast, externalization of phosphatidylethanolamine (PE) and phosphatidylserine (PS) may be more specifically involved in plasma membrane-derived EV secretion, and V-ATPase-mediated acidification of MVBs may be a specific control mechanism of exosome secretion, although V-ATPase inhibitors may also affect the Golgi V-ATPase …

A key limitation for the precise characterization of EVs has been the technical difficulty in isolating and characterizing pure populations of specific subtypes, as the methods currently at our disposal lead to the systematic co-isolation of EVs of distinct subcellular origins. Thus, although many articles use the term 'exosome' to refer to EV preparations that have been separated from larger EVs by physical processes, it is likely that they rather refer to a mixture of small EVs of both exosomal and non-exosomal nature. Hence, unless their MVB origin has been clearly established, it may be preferable to favour the generic term 'small EVs'118.

The authors then continue to a critical point. Namely the delivery process and mechanism. There are two issues: (i) the movement of the exosome through the circulatory system and its selection of an exit point, (ii) the attachment and deposition of its cargo in a target cell. We have understanding of such in a multiple of other cells but none quite like what we would see in an exosome and miRNA system.

Our current knowledge of EV physiology, diversity, internalization and cargo delivery is still too limited to yield clear mechanistic conclusions about precisely how EVs interact with and modify acceptor cells. For the EV field to progress, it will be necessary to perform studies in a comprehensive manner that includes molecular, cellular and functional characterization and, to the extent possible, also compares different EV subtypes in a given experimental system. Such approaches will be crucial to determine which of the identified molecules or mechanisms are specific to certain EV subtypes versus those that are instead applicable to all EVs. A specific area that would benefit from further investigation is EV uptake. There is currently no consensus regarding the main route followed by EVs or a given EV subtype to deliver content in the cytosol of acceptor cells.

They then discuss the internalization issue. We would consider this as more of a targeting issue. The question here may be; is there a packaging of exosomes with miRNAs and ligands targeted at a collection of cells? This would be a complex process and would be much more than what we seen in most malignant proliferations. The authors continue:

Determining whether internalization occurs through macropinocytosis or micropinocytosis and/ or receptor-mediated pathways, and whether these processes result in cargo delivery will be essential to understand and control EV uptake. A suitable approach may be to first track a generic soluble cargo present in many different EV types to determine the conditions (including donor and acceptor cell combinations) under which cargo exchange has notable physiological and biochemical effects. This knowledge would then permit characterization of the cellular and molecular underpinnings of EV uptake and content delivery. It will also be important to consider how the various biogenesis, release and uptake pathways affect intracellular functions not related to EVs.

Such systematic analyses will facilitate the identification of specific EV functions in physiological and pathological settings, and will aid in translating this knowledge for the treatment of pathological effects of EVs or the therapeutic application of EV-relevant cellular mechanisms.

Clearly the exosome and EV knowledge is still formative but highly suggestive.

3 METASTASIS

Metastasis is often the final stage of many cancers. It is the promulgation of malignant cells to distant locations. Just how this is accomplished is still shadowed in uncertainty. The recent book by Lyden et al presents a comprehensive overview of current understanding. However there are many yet to answered issues. In this section we briefly compare the Classical and what we have called the Neo-Classical views.

3.1 THE CLASSIC VIEW

A classic view still holding states (see Mostoslavsky and Bardeesy):

…studies are conceptually appealing, indicating that the metastatic program involves a resetting of the cell to a more primitive, developmentally plastic, differentiation state, driven by a largescale change in the active enhancer landscape. This work raises a number of interesting questions. First, while enhancers are known to be important for specifying transcriptional programs that drive cell fate changes, it is intriguing that a large-scale shift of enhancer usage is particularly critical for PDA metastasis.

It remains to be explored whether this phenomenon plays a role in other cancer types and, if so, whether it might involve similar themes of pioneer transcription factor recruitment and partial reversion to earlier stages of differentiation. Second, it is worth noting that in addition to the enhancer signature, the M organoids showed a key genetic alteration that distinguished them from T organoids, namely specific loss of the remaining wild-type allele of p53, which likely contributes to the metastatic phenotype. While the authors show that p53 inactivation in T organoids does not acutely induce Foxa1 mRNA expression or provoke the Foxa1- driven enhancer signature, it is possible that in vivo p53 loss could create a permissive setting for Foxa1 activation.

Overall, the program controlled by p53 in this context, as well as the molecular switch that underlies initial Foxa1 induction, warrants investigation. There are a number of mechanistic questions that also emerge from these studies. For example, the authors report that their metastatic models did not exhibit a typical epithelial-to-mesenchymal (EMT) transition, which has previously been implicated in metastasis.

Metastatic cancers often give off circulating tumor cells. These CTC can be found in the blood stream as well as various EVs. As Gkountela et al note:

Circulating tumor cells (CTCs) are those cells that depart from cancerous lesions and enter the bloodstream. Although extraordinarily rare compared with blood cells and forced to strive for survival in circulation, CTCs are considered to be precursors of metastasis in various cancer types, including breast cancer. CTCs are found in the blood of cancer patients as single CTCs and CTC clusters , with the latter featuring a higher ability to seed metastasis.

However, it is unknown what drives their enhanced metastatic potential and what are the vulnerabilities of clustered CTCs. Abnormal DNA methylation patterns, including both genomewide hypomethylation and hypermethylation, have been associated with several human cancers.

Generally, these cancer-associated epigenetic modifications appear to affect distinct genomic areas, with hypomethylation favoring regulatory and repetitive elements versus hypermethylation, which is more frequent in CpG islands. Both modifications have the ability to alter the expression of neighboring genes and contribute to the cancer phenotype.

Epigenetic factors are also critical. We have discussed epigenetic factors from prostate cancers to MDS hematopoetic cancers. The authors then consider this as well:

For regulatory elements, loss of DNA methylation at transcription factor binding sites (TFBSs) can designate active transcription factor networks or networks primed for activation at later stages, e.g., during processes such as the derivation of induced pluripotent stem cells from differentiated cells or cancer progression.

Although DNA methylation analysis of primary tumors is extensively investigated, the forces that shape the DNA methylome during metastatic dissemination are largely uncharacterized.

Here, we combine microfluidic-based CTC capture from breast cancer patients and mouse models, single-cell resolution DNA methylation and RNA expression analysis, a drug screen with 2,486 FDA-approved compounds, and functional validation studies in mouse models to gain insights into the biology and vulnerabilities of CTC clusters. Our study provides a genome-wide DNA methylation landscape of single and clustered CTCs in breast cancer, highlighting fundamental differences that affect metastasis and enabling the identification of cluster-targeting compounds with immediate clinical applicability.

They conclude by stating:

Our study provides a comprehensive genome-wide analysis of the DNA-methylation events that characterize CTCs in patients and xenografts. Surprisingly, we find that phenotypic differences— such as the ability of CTCs to navigate through the bloodstream as single cells or multicellular clusters—shape the DNA methylome.

Clustering of CTCs results in hypomethylation of binding sites that are typically occupied by master stemness and proliferation regulators, including OCT4, NANOG, SOX2, and SIN3A, and hypermethylation of Polycomb target genes. More globally, we also find that the DNA methylation profile of CTC clusters is detected at the level of the primary tumor in a subset of breast cancers that are characterized by a poor prognosis. CTC clusters dissociation into single cells with Na+/K+ ATPase inhibitors or through cell-cell junction knockdown enables DNA methylation remodeling at critical sites, highlighting a direct connection between clustering and methylation status. As a result, Na+/K+ ATPase inhibitors treatment emerges as a new strategy to significantly reduce the spread of cancer, providing a rationale for using these compounds in clinical studies.

Our results suggest that CTC clusters may share several properties that commonly feature stem cell biology. For instance, OCT4, NANOG, SOX2, and SIN3A are predominantly active in embryonic stem cells (ESCs), simultaneously regulating selfrenewal and proliferation. In addition, ESCs rely on Polycomb-mediated repression of differentiation genes and chromatin remodeling to maintain their active pluripotency network.

Cell-cell junction activity has been shown in several instances to safeguard pluripotency and to be required for a complete reprogramming of somatic cells into stem cells, and disruption of cell-cell junctions (e.g., through targeting of E-cadherin) in human ESC results into OCT4, NANOG, and SOX2 downregulation along the loss of stemness features. Thus, by analogy with stem cell biology, elevated expression of cell-cell junction components in cancer cells may not only enable their intravasation in the bloodstream as multicellular clusters but also their ability to retain stem-like features that facilitate metastasis initiation.

3.2 THE NEO-CLASSIC EXOSOME VIEW

We now consider some of the research which give some credence to the Neo Classical view. We begin with the work of Mehlen and Puisieux who have noted regarding metastasis:

Metastasis occurs through a series of sequential steps in which tumour cells first migrate from the primary tumour, penetrate blood vessels and then colonize distant sites. It is a highly inefficient process. Indeed, very few of the tumour cells that gain access to the vasculature give rise to metastastic foci in a secondary organ. Recent data indicate that the mechanisms controlling metastasis can be regulated independently from primary tumour development. In vitro and in vivo, the metastatic potential of tumours is associated with an increased resistance to apoptosis.

Furthermore, the experimental modulation of apoptotic or anti-apoptotic factors influences metastatic efficiency. Anoikis and amorphosis are important barriers to metastasis. Anoikis is cell death induced by the disruption of cell attachment and cell–matrix interactions, whereas amorphosis is cell death stimulated by the loss of cytoskeletal architecture. Early survival of tumour cells after attachment to the secondary site and the development of micrometastases are crucial steps of the metastatic process.

Metastasis is the most common cause of cancer death. Most patients with metastatic disease respond transiently to conventional treatments. Further elucidation of the relationship between resistance to apoptosis of metastatic cancer cells and their chemoresistance should provide important clues to improve systemic therapies.

The authors follow through with the construct of physical malignant tumor movement. They note:

To enter the bloodstream a tumour cell has to negotiate and survive the process of intravasation. A fluorescence- based in vivo study using orthotopically injected metastatic versus non-metastatic tumour cells showed that a large number of the non-metastatic cells fragment when interacting with blood vessels, whereas the metastatic cells display increased survival during

this process, indicating that the entry of cancer cells into the vasculature constitutes an important barrier to metastasis.

Furthermore, to form metastases, circulating cancer cells have to pass through several stressful and highly selective steps, including survival in the bloodstream, arrest in the capillary bed and resumption of proliferation in distant organs. What happens to the vast majority of the tumour cells that fail to form metastases is still matter of debate. Initial evidence, based on radiolabelled cancer lines injected into the circulation, indicated that most cells died either in the blood vessels or rapidly after extravasation.

Although the extent of such cell death might be significantly influenced by the experimental model, more recent reports, based on the injection of fluorescent-labelled cancer cell lines and intravital videomicroscopy in mice, support the hypothesis that solitary cancer cells in the circulation, or soon after extravasation, are sensitive to apoptosis.

In line with this assumption, apoptosis resistance owing to BCL2 overexpression is associated with a tenfold decrease in the number of apoptotic tumour cells at the secondary site 1 hour after intravenous injection, and with an increase in metastasis formation. Most of the studies reported here are based on the injection of cells in rodents and these experiments use metastatic cancer cell lines that, by definition, have somehow already acquired apoptosis resistance to some extent. Therefore, the efficiency of metastasis inhibition by apoptosis induction in the early phases of entrance into the circulation and extravasation is probably an important regulatory mechanism that counteracts metastasis development.

Cell death might occur as a result of two main effects: cell destruction by mechanical stress and cell death mediated by the immune system.

But what if movement of the whole malignant cell is not necessary? What if the only element required is some activating miRNA which is encapsulated in an exosome? Then metastasis can occur more rapidly and efficiently. Furthermore the cell of origin or the cancer stem cell may sty just where it is, eluting exosomes to the body.

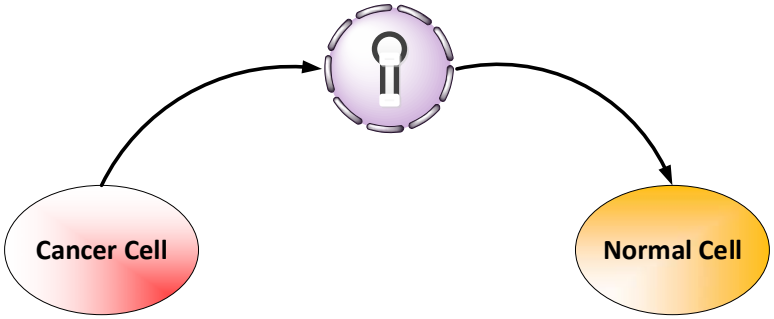
Now we know that exosomes can promote metastasis. As Zaborowski et al have noted:

Neoplastic cells can also release EVs that modify the phenotype of host cells to facilitate tumor growth. EVs released from ovarian cancer cells contain CD147, which promotes the expression of MMP-1, -2, and -9 in endothelial cells. EVs promote angiogenesis by stimulating the migration and tubule formation of endothelial cells. Interestingly, this effect was also exerted by EVs derived from renal cancer stem cell populations and therefore may be a common property of cancer cell-derived EVs.

The angiogenic activity was stronger if EV-producing cells were cultured under hypoxic conditions. EGFR transferred to endothelial cells from cancer cells via EVs induced the autocrine release of vascular endothelial growth factor to support angiogenesis. Oncosomes were also shown to trigger the migration of cancer-associated fibroblasts. Interestingly, CAFs, in turn, shed EVs with a high content of miR-409, which contributed to the EMT transition and high cancer stem cell phenotypes.

EVs released by ovarian cancer cells contributed to the expansion and higher functional competence of regulatory T lymphocytes and the apoptosis of cytotoxic T lymphocytes that results in the suppression of antitumor immune responses. The incubation of breast cancer- or glioma-released EVs with fibroblasts and epithelial cells resulted in the increased anchorage-independent growth and survival of host cells, suggesting features of transformation in nonneoplastic host cells. Taken together, the exchange of EVs between cancer and normal cells in the tumor microenvironment can result in the promotion of tumor growth through multiple mechanisms.

We graphically demonstrate this below where we show the cancer cell transmitting an exosome with an miRNA to a normal cell.



As Becker et al have noted:

Tumor-secreted EVs are emerging as critical messengers in tumor progression and metastasis. In this review, we summarize the metastatic role of various EVs: microvesicles, exosomes, ectosomes, oncosomes, etc.. Exosomes are EVs that are 30–150 nm in diameter and derived from the multivesicular endosome pathway, but the term is used in many studies for small EVs recovered by various protocols that do not actually discriminate endosome-derived from plasma membrane-derived EVs.

We thus use the term as chosen by the authors of the articles described, not necessarily inferring an exclusively endosomal or plasma membrane origin of the EVs. EVs contain bioactive molecules, such as nucleic acids (DNA, mRNA, microRNA [miRNA], and other non-coding RNAs), proteins (receptors, transcription factors, enzymes, extracellular matrix proteins), and lipids that can redirect the function of a recipient cell.

Cancer cell-derived EVs promote angiogenesis and coagulation, modulate the immune system, and remodel surrounding parenchymal tissue, which together support tumor progression. Clinically, circulating exosomes and microvesicles isolated from cancer patients have been associated with metastasis or relapse, and therefore could serve as important diagnostic and prognostic markers as well as therapeutic targets.

They continue:

Tumor EVs exert complex effects on neighboring stromal cells, such as endothelial cells and fibroblasts. Glioblastoma-derived microvesicles containing mRNA, miRNA, and angiogenic proteins are taken up by recipient cells and promote primary tumor growth as well as endothelial cell proliferation. Pancreatic cancer-derived exosomes expressing tetraspanin 8 recruit proteins and mRNA cargo that activate angiogenesis- related gene expression in endothelial cells.

Tumor-derived exosomes containing transforming growth factor b (TGF-b) convert fibroblasts into myofibroblasts, contributing to vascularization, tumor growth, and local invasion. Breast cancer-derived exosomes also promote a myofibroblastic phenotype in adipose tissue-derived mesenchymal stem cells, resulting in increased expression of the tumor-promoting factors TGF-b, vascular endothelial growth factor (VEGF), stromal cell-derived factor 1 (SDF-1), and C-C motif chemokine ligand 5 (CCL5).

Conversely, exosomes secreted by tumor stroma can also influence tumor progression. Breast-cancer-associated fibroblasts secrete exosomes that have been shown to promote tumor motility, invasion, and dissemination of breast cancer cells through the Wnt-planar cell polarity (Wnt-PCP) signaling pathway. Therefore, exosomes mediate bidirectional communication between tumor cells and their environment and are central effectors of a feedforward signaling loop that shapes the ever-evolving tumor microenvironment.

The following discussion presents an interesting question. Namely the interaction between normal calls and malignant ones via exosomes. Perhaps the exosomes can also capture the ability of a normal cell to enhance metastatic behavior as well. This is discussed by the authors as follows;

However, the specific mechanisms through which healthy stromal cells are triggered to release exosomes that promote the malignant behavior of cancer cells remain to be determined. In the past decade, much emphasis has been placed on the potential role of miRNAs packaged within EVs in regulating cell-cell interactions. Exosomes released by mast cells containing both mRNA and miRNA can be transferred to recipient cells and regulate gene expression. Moreover, transfer of miRNAs specifically targeting PTEN expression from astrocyte-derived exosomes to invading tumor cells in the brain microenvironment promotes establishment of brain metastasis, although other autocrine and paracrine signaling may also cooperate during tumor progression.

However, the significance of this horizontal transfer of miRNAs for the global miRNA activity of a target cell remains unclear detected transfer of miRNA activity via exosomes but the contribution to target gene repression was limited, suggesting exosomal miRNAs are degraded within recipient cell lysosomes. While these studies were performed in endothelial cells, other microenvironments, such as primary tumors, may facilitate increased miRNA transfer among cells.

Recently, it was suggested that cancer exosomes, on average, contained only a single miRNA per exosome. However, stoichiometry of specific miRNAs may vary between tumor types, and

therefore the number and distribution of miRNAs secreted in other models may differ from these studies.

The last remark is of interest. If it is true that the exosome contains a single miRNA then it raises the question of selective exosome formation and in turn the establishment of target ligands on the exosome surface. Is there a much more complex process at work here?

Metastasis is a complex process. We have presented a new approach which as we have noted has some basis in factual observation. However it is still highly conjectural and a great deal more needs to be accomplished.

4 MIRNA

We provide a brief overview of miRNAs. Ther are for the most part small sections of RNA, usually about 22 nucleotides, and they are also found with a loop portion in the middle.

As Melo et al have noted:

MicroRNAs (miRNAs) are small non-coding RNAs of 18–24 nucleotides (nt) in length that control gene expression post-transcriptionally. They are synthesized via sequential actions of Drosha and Dicer endonucleases, and incorporate with the RNA induced silencing complex (RISC) to target mRNAs.

RISC-loaded miRNAs bind in a sequence-specific manner to target mRNAs, initiating their repression through a combination of translational inhibition, RNA destabilization or through direct RISC-mediated mRNA cleavage.

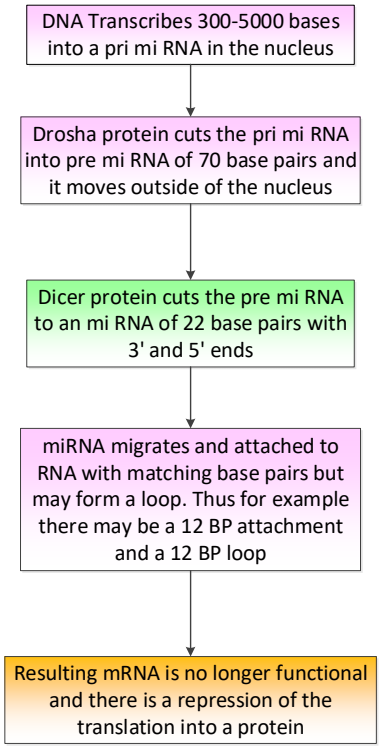
For a miRNA to be functional and achieve efficient gene silencing, it must form a complex with the RLC (RISC-loading complex) proteins Dicer, TRBP, and AGO2. Within the RLC, Dicer and TRBP process precursor miRNAs (pre-miRNAs) after they emerge from the nucleus via exportin-5, to generate miRNAs and associate with AGO2. AGO2 bound to the mature miRNA constitutes the minimal RISC and may subsequently dissociate from Dicer and TRBP.

Single-stranded miRNAs by themselves incorporate into RISC very poorly and therefore, cannot be efficiently directed to target mRNAs for post-transcriptional regulation. Nonetheless, several reports suggest that miRNAs contained in exosomes can influence gene expression in target cells.

Drosha and Dicer are present in exosomes from cell culture supernatants from HIV-1 infected cells and HIV patient sera. Co-fractionation of Dicer, TRBP and AGO2 in late endosome/MVB (multivesicular body) is also observed. These studies reflect the need to evaluate the functional contribution of miRNA machinery proteins in exosomes and their role in tumor progression.

4.1 MIRNA FUNCTIONING

Now we will details some of this below;



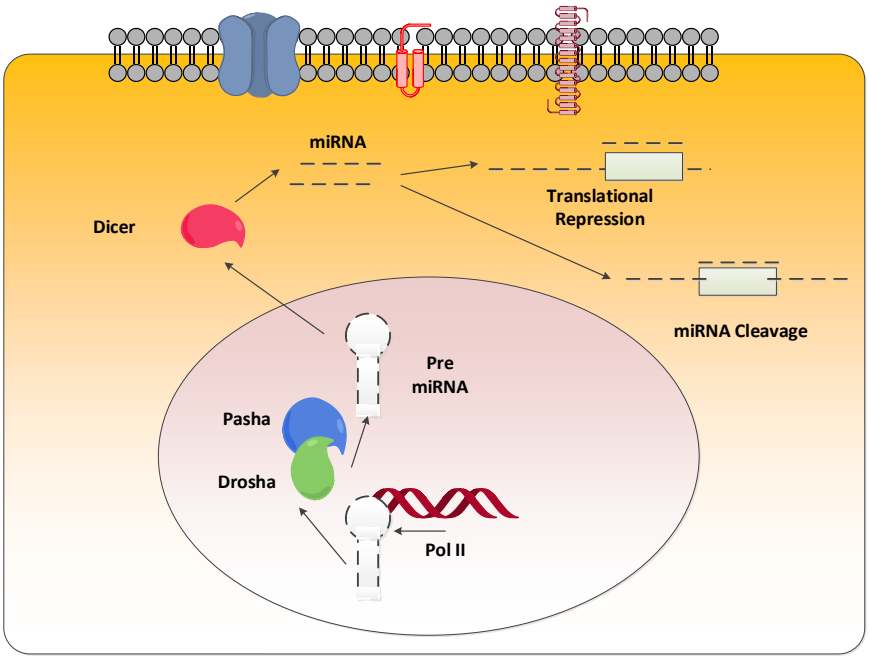
As Lou et al note:

MiRNAs are non-coding, small, single-stranded RNAs that are derived from the primary transcript called pri-miRNA, which is transcribed by RNA polymerase II. The pri-miRNAs are characterized by the presence of a single or multiple imperfect hairpin structures with a stem of approximately 33 base-pairs. Subsequently, the primiRNA precursor undergoes a two-step processing pathway, mediated by two ribonucleases, Drosha and Dicer belonging to the RNase III family.

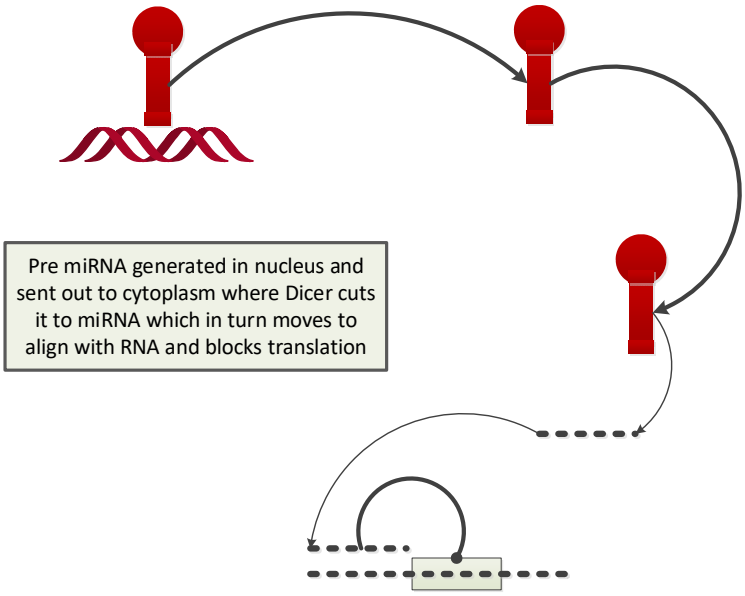
In the nucleus, Drosha cleaves the pri-miRNA to generate an approximately 70 nucleotides long pre-miRNA, which is exported to the cytoplasm via an exportin-5-dependent mechanism. In the cytoplasm, the pre-miRNA is further processed by Dicer to generate a mature, functional, double-stranded miRNA.

Then, the guide strand or mature miRNA is integrated into a multi-protein complex, RISC, which contains the argonaute (AGO) protein that plays a central role in RNA silencing. RISC uses the guide strand to target complementary 3'-UTR of mRNA via Watson- Crick base pairing. The other strand which is known as miRNA or passenger strand is eventually degraded. The miRNA binding to the 3'-UTR leads to mRNA degradation or translational repression, the extent of which is dependent on the degree of complementation. Besides, RISC can also target 5'-UTR of mRNA and activate translation.*

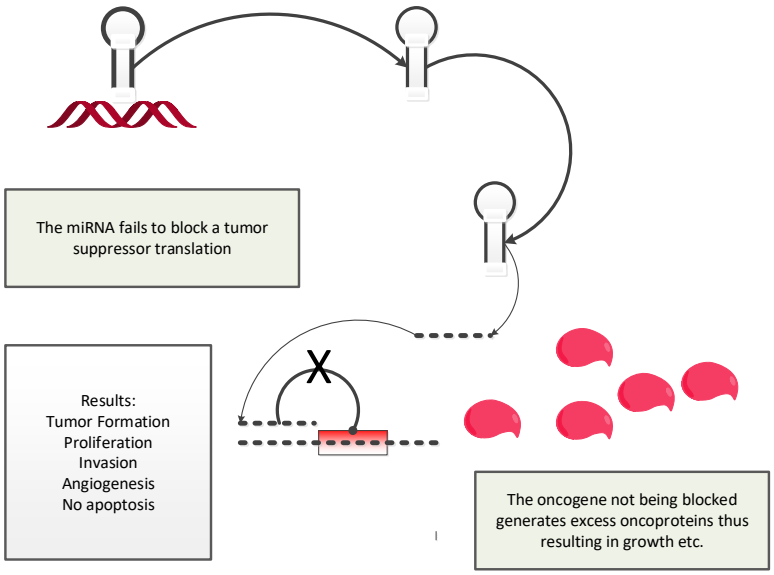
We characterize this in the Figure below.



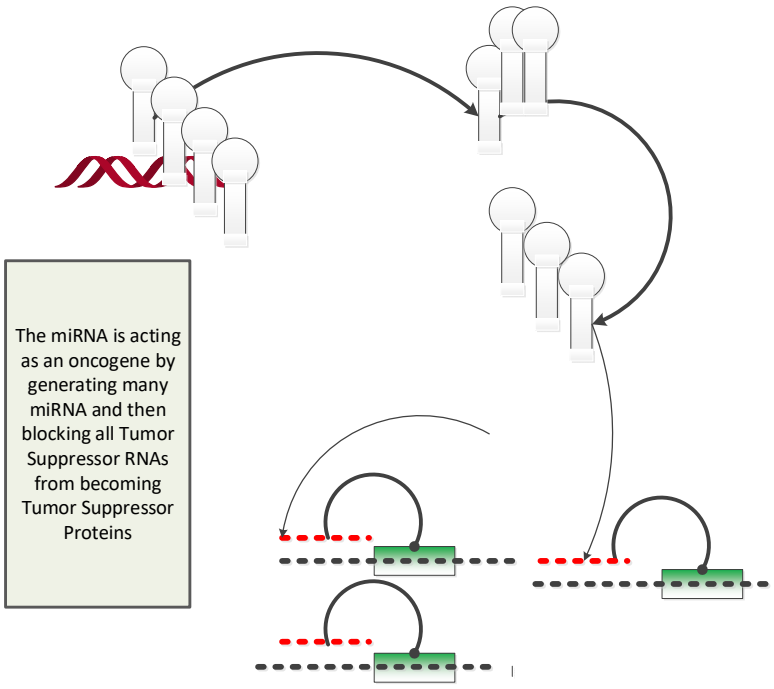
We can now briefly go through the process of generating an miRNA. First is the generation of the pre miRNA through the work os Pasha and Drosha. It sends this out to the cytoplasm.



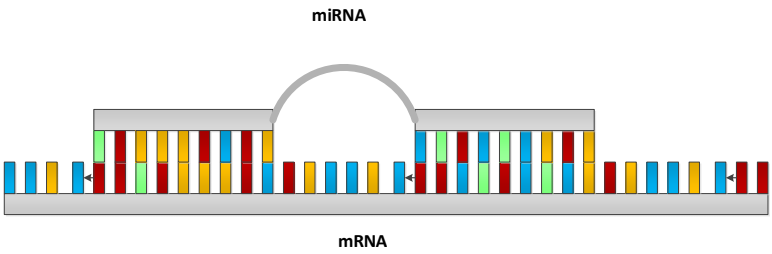
Then in the cytoplasm we get it cut and ready to be applied. It manages to attach itself to a mRNA and in so doing blocks the expression of the gene from whence it came.



They can then multiply across the cell and acts as an oncogene as shown below.



Specifically it matches up to a mRNA as shown below and blocks it transcription.



Now this blockage is across a specific 20 base area with a gap from the hinge in the middle. This can be a fairly specific target.

4.2 miRNA and Cancer

There is now a great deal of evidence of the impact of miRNAs and cancer development. As Lou et al note:

Vascular endothelial growth factor (VEGF) consists of VEGFA, VEGFB, VEGFC, VEGFD and placenta growth factor (PGF). Ectopic expression of VEGF partly accounts for cancer progression because of its involvement in cancer angiogenesis and metastasis. Many miRNAs regulate the VEGF expression. MiRNA-29c overexpression inhibits angiogenesis by downregulating VEGF. Moreover, upregulation of miRNA-29c suppresses in vitro glioma cell migration and invasion due to reduced MMP-2 levels. Wang et al. reported that the low expression of miRNA-195 promotes angiogenesis and metastasis of HCC via VEGF and the pro-metastatic factors, VAV2 and CDC42.

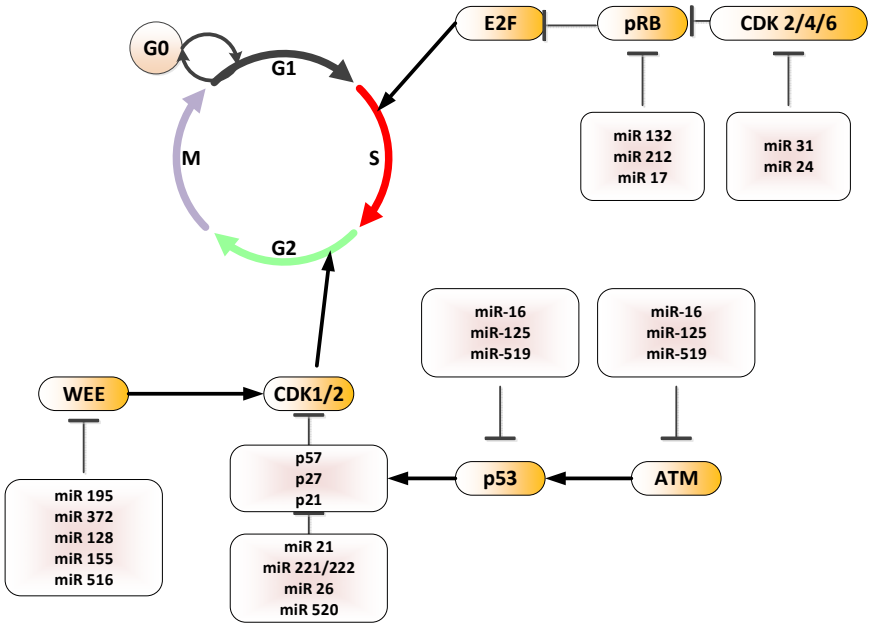
Ghosh et al. showed that miRNA-199a-3p was downregulated in HCC tissues; its overexpression suppressed cancer growth, angiogenesis and lung metastasis by suppressing VEGFA, VEGFR1, VEGFR2, HGF and MMP2. Tu et al. showed that miRNA-497 inhibited breast cancer angiogenesis by targeting VEGFR2. Twist-induced downregulation of miRNA-497 promoted angiogenesis and metastasis of pancreatic cancer and was associated with high levels of VEGFA. Besides, miRNA-497 suppressed HCC angiogenesis and metastasis by inhibiting VEGFA.

We will examine these relationships in some detail to demonstrate these effects.

4.3 miR and Cell Cycle and Apoptosis

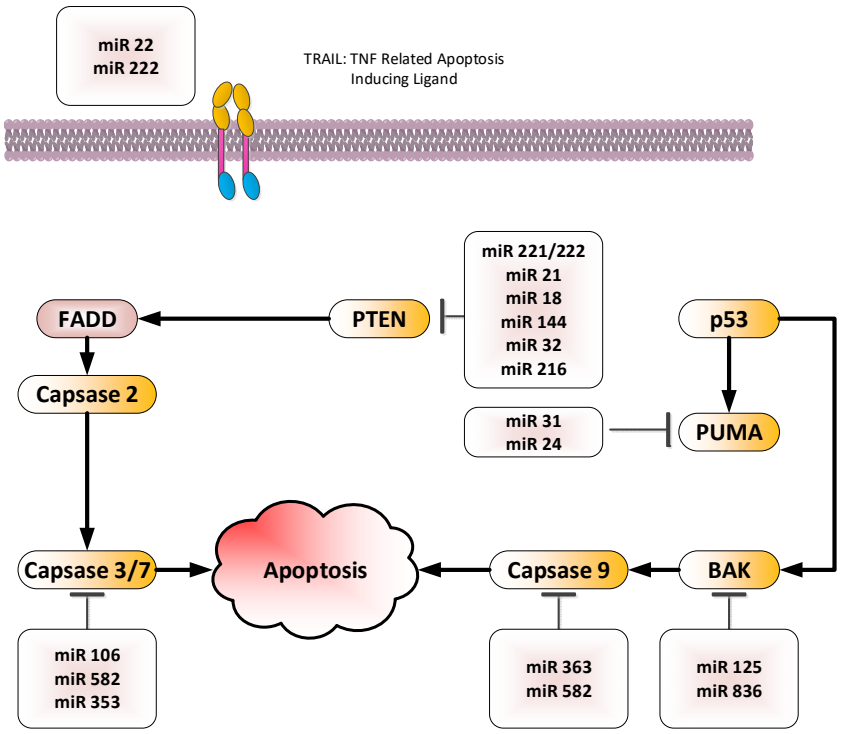
Cell proliferation and death are two elements strongly influenced by miRNAs. Proliferation is the main stay of a metastatic environment and apoptosis is its balancing alternative. Understanding the complex interplay with miRNAs is essential.

The cell cycle leading to proliferation is shown below[2]. Note the multiplicity of regulating miRNAs. Some block via other genes and some promote. This is a complex interaction and the ordering of the importance of the various miRNAs is open to consideration



Cell death, apoptosis is shown below again with a multiplicity of miRNAs. Again note the complexity of interactions and the need to have an ordering of the impacts of the miRNAs.

[2] We use the reference *Oncogenic MicroRNAs: Key Players in Malignant Transformation* by Tania Frixa, Sara Donzelli and Giovanni Blandino



The above is somewhat all inclusive. Namely many miRNA has been referenced. The question is: which miRNA is controlling and why? Clearly miRNA control in some fashion proliferation and cell demise. Ranking them is a critical factor.

It would be interesting to model these above two types of behavior. The problem is that at best we have putative causation via blockage. Again the issue rests in the details of the dynamics, none of which we seem to have at present.

5 HISTONES AND SIRT1

We now will examine SIRT1 and the family of genes from which it derives, the Sirtuins. These genes have generally been examined in many venues (see Rahman and Islam). This examination considers the impact of miRNAs, cancerogenesis, metastasis, and more importantly the epigenetic impacts. This examination is a benchmark for many other specific and complex genes associated with cancers. As Rahman and Islam initially note:

Sirt1 (member of the sirtuin family) is a nicotinamide adenosine dinucleotide (NAD)-dependent deacetylase that removes acetyl groups from various proteins. Sirt1 performs a wide variety of functions in biological systems. The current review focuses on the biological functions of Sirt1 in obesity-associated metabolic diseases, cancer, adipose tissue, aging, cellular senescence, cardiac aging and stress, prion-mediated neurodegeneration, inflammatory signaling in response to environmental stress, development and placental cell survival.

SIRT1 has many functions and it is a critical gene which can be controlled my miRNAs. Thus is presents an example of how exosome flow can result in massive gene control. SIRT1 based upon NCBI. From NCBI we have for SIRT1[3]:

SIRT1: This gene encodes a member of the sirtuin family of proteins, homologs to the yeast Sir2 protein. Members of the sirtuin family are characterized by a sirtuin core domain and grouped into four classes. The functions of human sirtuins have not yet been determined; however, yeast sirtuin proteins are known to regulate epigenetic gene silencing and suppress recombination of rDNA. Studies suggest that the human sirtuins may function as intracellular regulatory proteins with mono-ADP-ribosyltransferase activity. The protein encoded by this gene is included in class I of the sirtuin family. Alternative splicing results in multiple transcript variants.

The regulatory nature of SIRT1 is a key element in its functioning in Prostate Cancer and many other cancers. We will examine how this may function shortly.

In addition, SIRT1 is frequently controlled by miRNAs as noted by Eades et al. Their focus was on breast cancer where they have remarked:

Evidence supports a critical role for microRNAs (miRNAs) in regulation of tissue-specific differentiation and development. Signifying a disruption of these programs, expression profiling has revealed extensive miRNA dysregulation in tumors compared with healthy tissue. The miR-200 family has been established as a key regulator of epithelial phenotype and, as such, is deeply involved in epithelial to mesenchymal transition (EMT) processes in breast cancer. However, the effects of the miR-200 family on transformation of normal mammary epithelial cells have yet to be fully characterized.

By examining a TGF-β driven model of transformation of normal mammary epithelium, we demonstrate that the class III histone deacetylase silent information regulator 1 (SIRT1), a

[3] http://www.ncbi.nlm.nih.gov/gene/23411

proposed oncogene in breast cancer, is overexpressed upon EMT-like transformation and that epigenetic silencing of miR-200a contributes at least in part to the overexpression of SIRT1. We have established the SIRT1 transcript as subject to regulation by miR-200a, through miR- 200a targeting of SIRT1 3 UTR. We also observed SIRT1 and miR-200a participation in a negative feedback regulatory loop. Restoration of miR-200a or the knockdown of SIRT1 prevented transformation of normal mammary epithelial cells evidenced by decreased anchorage-independent growth and decreased cell migration.

Finally, we observed SIRT1 overexpression in association with decreased miR-200a in breast cancer patient samples. These observations provide further evidence for a critical tumor suppressive role of the miR-200 family in breast epithelium in addition to identifying a novel regulatory mechanism, which may contribute to SIRT1 up-regulation in breast cancer.

This is just one of many examples of SIRT1, histone control, and miRNAs and various cancers. We have examined this in prostate, breast, melanoma, and a variety of other malignancies. We also note the focus on EMT which we have also examined previously. EMT is a major first step in metastatic transitions. The cells start to move and obtain the flexibility to disassociate themselves from their primary functional locations.

From Powell et al we have as more detailed discussion of the functions of Sirt1:

The Sirtuin family of proteins (SIRT) encode a group of evolutionarily conserved, NAD-dependent histone deacetylases, involved in many biological pathways. SIRT1, the human homologue of the yeast Silent Information Regulator 2 (Sir2) gene, de-acetylates histones, p300, p53, and the androgen receptor. Autophagy is required for the degradation of damaged organelles and long-lived proteins, as well as for the development of glands such as the breast and prostate. Herein, homozygous deletion of the Sirt1 gene in mice resulted in prostatic intraepithelial neoplasia (PIN) associated with reduced autophagy.

Genome-wide gene expression analysis of Sirt1/ prostates demonstrated that endogenous Sirt1 repressed androgen responsive gene expression and induced autophagy in the prostate. Sirt1 induction of autophagy occurred at the level of autophagosome maturation and completion in cultured prostate cancer cells. These studies provide novel evidence for a checkpoint function of Sirt1 in the development of PIN and further highlight a role for SIRT1 as a tumor suppressor in the prostate.

The autophagy cleans up the cells and brings them back to a normal stasis. The recognition of Powell et al regarding the role of Sirt1 is key. They continue:

The role of SIRT1 in regulating prostate gland formation and androgen signaling in vivo was previously unknown. SIRT1is expressed in several cell types in the prostate gland including basal cells, luminal cells, and stromal cells. Given the evidence that SIRT1 functions as a tissue-specific regulator of cellular growth and that SIRT1 inhibits tumor cell line growth in nude mice, we sought to determine the role of endogenousSirt1 in regulating prostate gland development. Genome-wide expression profiling of Sirt1/ mice prostates and their littermate controls identified a molecular, genetic signature regulated by endogenous Sirt1.

The above clearly shows the understanding of the function of Sirt1. Note that the Powell work was in 2010 so that this understanding has been available for a while.

This signature highlights the ability of Sirt1 to inhibit androgen signaling and apoptosis in the prostate, while promoting autophagy. The Sirt1/ prostates demonstrated epithelial hyperplasia and PIN suggesting that Sirt1 promotes autophagy and inhibits prostate epithelial cell proliferation in vivo.

The above demonstrates the ability of Sirt1 to control androgen signalling. This also is a key factor in controlling prostate health.

Gene expression analysis further demonstrated that loss of endogenous Sirt1 inhibited autophagy. At a higher level of resolution, our studies demonstrated that SIRT1 antagonized DHT-mediated inhibition of autophagy in the prostate. Autophagy allows for degradation of proteins and organelles and is induced by nutrient withdrawal, rapamycin (inhibition of mTOR signaling), and hormone signaling.

Our findings are consistent with prior studies demonstrating that SIRT1 induces autophagy by deacetylating ATG5, ATG7, and ATG8 and inhibits AR signaling via deacetylation of the AR. Comparisons with previously published studies identified an overlap of 12.45% between genes regulated by endogenous Sirt1 and those targeted by androgens in the prostate gland and in prostate cancer cells. These results are consistent with prior findings that Sirt1 inhibits ligand-dependent AR signaling and gene expression in vitro

Again we come back to the role of autophagy. Perhaps the buildup of protein segments may act as normal cell blockage, inhibiting normal expression and control. The autophagy allows for a return to such normality. The emphasize this issue as follows:

The role of autophagy in cancer was proposed over 20 years ago. Autophagy appears to be essential for tumor suppression as well as for cell survival. Autophagy plays a prosurvival function for cancer cells during nutrient deprivation or when apoptotic pathways are compromised, a phenotype often accompanied by inflammation.

Again we see the putative role of inflammation. This appears to be a significant factor in PCa and the suppression of genes which deal with the remnants of inflammation seem to be a key benchmark in PCa progression. They continue:

In contrast, upon disruption of tumor suppressors, autophagy adopts a pro-death role with apoptotic pathways. In prostate, breast, ovarian, and lung cancer, loss of Beclin1 or inhibition of Beclin1 by the BCL-2 family of proteins causes defective autophagy, increased DNA damage, metabolic stress, and genomic instability.

These cancers also display neoplastic changes and increased cell proliferation, unlike cells overexpressing Beclin1, which undergo apoptosis. Loss of PTEN, p53, ATG4, ATG5, and MAP1LC31 (ATG8) are linked to tumorigenesis, whereas upregulation of PI3K, AKT, BCL-2, and mTOR are associated with inhibition of autophagy and the promotion of tumorigenesis.

Prostate cancer onset and progression are correlated strongly with aging and SIRT1 function governs aging in multiple species. Further studies will be required to determine whether this checkpoint function of Sirt1 in regard to prostate growth is linked to its role in organismal aging.

From Shackelford et al we have additional insights including pathway control issues as follows:

AMPK has recently been shown to increase sirtuin 1 (SIRT1) activity by increasing cellular NAD+ levels, resulting in the regulation of many downstream SIRT1 targets, including FOXO3 and peroxisome proliferator activated receptor-γ co-activator 1 (PgC1; also known as PPARgC1A), both of which have also been proposed to be direct substrates of AMPK46,76. As SIRT1 is also implicated in tumorigenesis, this connection between AMPK and SIRT1 might further explain how nutrients control cell growth. AMPK also suppresses mTOR-dependent transcriptional regulators to inhibit cell growth and tumorigenesis.

Two mTORC1-regulated transcription factors involved in cell growth are the sterol-regulatory element-binding protein 1 (SReBP1) and hypoxiainducible factor 1a (HIF1α). SReBP1 is a sterolsensing transcription factor that drives lipogenesis in many mammalian cell types. mTORC1 signalling is required for nuclear accumulation of SReBP1 and the induction of SReBP1 target genes78, and this can be inhibited by rapamycin or AMPK agonists

From Hines et al we have an expression of Sirt1 in terms of overall cell control:

The NAD + -dependent deacetylase SIRT1 is an evolutionarily conserved metabolic sensor of the Sirtuin family that mediates homeostatic responses to certain physiological stresses such as nutrient restriction. Previous reports have implicated fluctuations in intracellular NAD + concentrations as the principal regulator of SIRT1 activity. However, here we have identified a cAMP-induced phosphorylation of a highly conserved serine (S434) located in the SIRT1 catalytic domain that rapidly enhanced intrinsic deacetylase activity independently of changes in NAD + levels.

Attenuation of SIRT1 expression or the use of a nonphosphorylatable SIRT1 mutant pre- vented cAMP-mediated stimulation of fatty acid oxidation and gene expression linked to this path- way. Overexpression of SIRT1 in mice significantly potentiated the increases in fatty acid oxidation and energy expenditure caused by either pharmacological b -adrenergic agonism or cold exposure. These studies support a mechanism of Sirtuin enzymatic control through the cAMP/PKA pathway with important implications for stress responses and maintenance of energy homeostasis

From Dominy et al we have:

From an evolutionary perspective, the nutrient-dependent control of protein acetylation through acetyltransferases and deacetylases is highly conserved and is a major mechanism for coupling metabolic activity with carbon/energy availability. The regulated acetylation of PGC-1a by GCN5 and Sirt1 is an excellent example: PGC-1a acetylation by GCN5 is favored under conditions of nutrient/energy abundance, whereas deacetylation by Sirt1 is favored under conditions of nutrient dearth and high energy demand

Finally, Brooks and Gu state:

SIRT1 is a multifaceted, NAD+-dependent protein deacetylase that is involved in a wide variety of cellular processes from cancer to ageing. The function of SIRT1 in cancer is complex: SIRT1 has been shown to have oncogenic properties by down regulating p53 activity, but recent studies indicate that SIRT1 acts as a tumour suppressor in a mutated p53 background, raising intriguing questions regarding its mechanism of action.

Here we discuss the current understanding of how SIRT1 functions in light of recent discoveries and propose that the net outcome of the seemingly opposite oncogenic and tumour-suppressive effects of SIRT1 depends on the status of p53.

They clearly indicate the tumor suppressor role of Sirt1. p53 status is important but the observation above is truly intriguing if it is sustained.

5.1 SIRT1 DETAILS

We begin with the work of Guatente has recently written an extensive review paper on Sirtuins and especially SIRT1 in NEJM. The studies to date have been on yeasts and fruit flies and there have been some studies on humans. However the main focus on sirtuins is their beneficial effects on the aging process, and one suspects as an antioxidant and anti-inflammatory type of behavior.

Of the mammalian sirtuins, SIRT1, 2, 3, 4, 5, and 6 have been shown to have this activity. Some SIRT family members (e.g., SIRT4 and SIRT6) also have ADP-ribosyltransferase activity. In mammals, the Sir2 orthologue SIRT1 is primarily a nuclear protein in most cell types and has evolved to deacetylate transcription factors and cofactors that govern many central metabolic pathways.

Targets of SIRT1 *include transcriptional proteins that are important in energy metabolism, such as nuclear receptors, peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α), and* ***forkhead box subgroup O (FOXO)****. SIRT1 also regulates components of the circadian clock, such as BMAL1 and PER2, which underscores the interconnectedness of protein acetylation, metabolism, circadian rhythm, and aging.*

SIRT1 is also closely coupled to AMP-kinase activity in a mutually enforcing mechanism that adjusts cellular physiology for conditions of energy limitation.

Sirt1 is the gene of focus yet Sirt2-6 also play roles, none of which seem to have a role in PCa. The FOXO target is of considerable interest[4].

[4] As Brunet et al state: S*IRT1's effects on FOXO3 are reminiscent of SIRT1's effects on the tumor suppressor p53. Under conditions of cellular stress, SIRT1 deacetylation of p53 leads to an inhibition of apoptosis. Given that SIRT1 also reduces FOXO3-induced apoptosis in the presence of stress stimuli, it is possible that FOXO3 and p53 somehow function together to mediate the effects of SIRT1.*We know p53 is an oncogene and its suppression can result in metastatic behavior and thus SIRT1 has a pivotal role in many areas of cancer development and spread.

The earliest connection between SIRT1 and endothelial cells was the finding that SIRT1 deacetylates and activates endothelial nitric oxide synthase (eNOS). The activation of eNOS and repression of AT1 suggest that SIRT1 activity ought to curb high blood pressure.

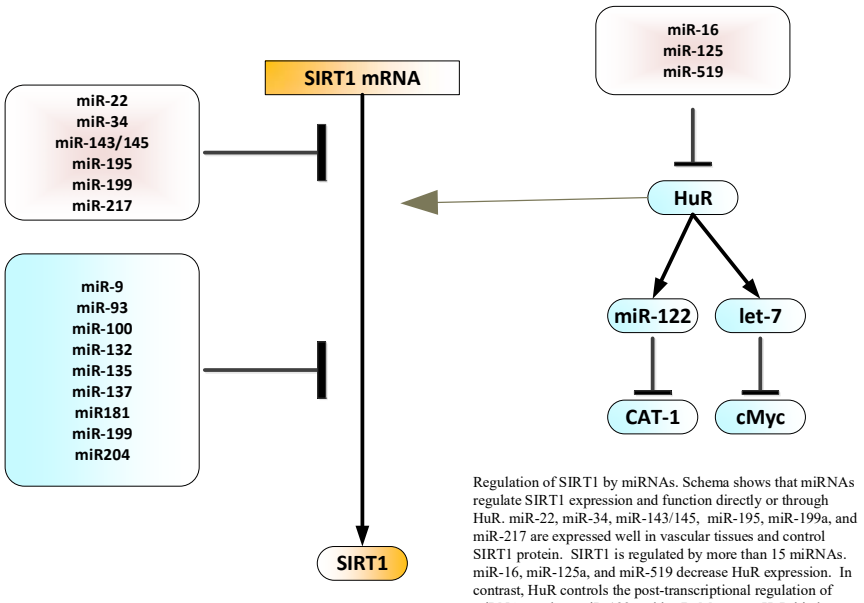
SIRT1 also inhibits the senescence of endothelial cells, and its salutary effect on these cells may mitigate atherosclerosis. Interestingly, calorie restriction is known to protect against atherosclerosis,46 and many of the physiological effects of calorie restriction are blunted in eNOS−/−mice.21 These findings all indicate that SIRT1 helps facilitate the favorable effect of calorie restriction on cardiovascular function by its effects on eNOS, AT1, and perhaps other targets.

Yamaguchi notes as follows additional nexus with miRNAs:

SIRT1 plays an important role in cancer. SIRT1 expression is increased in human cancers such as prostate cancer, colon cancer, acute myeloid leukemia, and some skin cancers . SIRT1 might act as a tumor promoter in these diseases by interacting with and inhibiting p53. SIRT1 also represses expression of tumor suppressor proteins and DNA repair proteins. But SIRT1 expression is decreased in other cancers, including ovarian cancer, glioblastoma, and bladder carcinoma. SIRT1 might serve as a tumor suppressor in these diseases by blocking oncogenic pathways. For example, SIRT1 limits β-catenin signaling in colon cancer, and in breast cancer BRCA1 signaling interacts with the SIRT1 pathway. Thus SIRT1 can serve as a tumor promoter or tumor suppressor, depending on the oncogenic pathways specific to particular tumors. …

SIRT1 signaling is complex, and controversies about the role of SIRT1 in longevity and cancer persist. Does SIRT1 really extend lifespan? Does SIRT1 accelerate or slow cancer progression? Regulation of SIRT1 adds to its complexity. Two major pathways for post-transcriptional regulations of SIRT1 exist, RBPs and miRNAs. A discrete set of miRNAs regulates SIRT1 expression, and different miRNA regulate SIRT1 in a cell specific manner. A major regulator of SIRT1 is miR-34a, which suppresses SIRT1 expression in specific tissues and cancers, including colon cancer. The RBP HuR is also involved in regulation of SIRT1 expression.

The interaction of SIRT1 and various miRNAs is shown below.



Regulation of SIRT1 by miRNAs. Schema shows that miRNAs regulate SIRT1 expression and function directly or through HuR. miR-22, miR-34, miR-143/145, miR-195, miR-199a, and miR-217 are expressed well in vascular tissues and control SIRT1 protein. SIRT1 is regulated by more than 15 miRNAs. miR-16, miR-125a, and miR-519 decrease HuR expression. In contrast, HuR controls the post-transcriptional regulation of miRNAs such as miR-122 and let-7. Moreover HuR binds to the 31UTR of SIRT1 and changes SIRT1 mRNA stability. See Yamakuchi

Now Knight et al also have noted regarding SIRT1 and cancer:

We have demonstrated a cancer-specific role for AROS in the regulation of survival in a panel of human cell lines. The data suggest that AROS, as well as SIRT1, promotes survival in cancer cells while being redundant for viability in non-cancer cells. However, at the molecular level, the roles of SIRT1 and AROS differ with respect to regulation of p53. We find evidence supporting a suppressive role for AROS in regulation of p53, as previously reported, but also that AROS function can be suppressed with no effect on p53—which is the case under basal conditions[5].

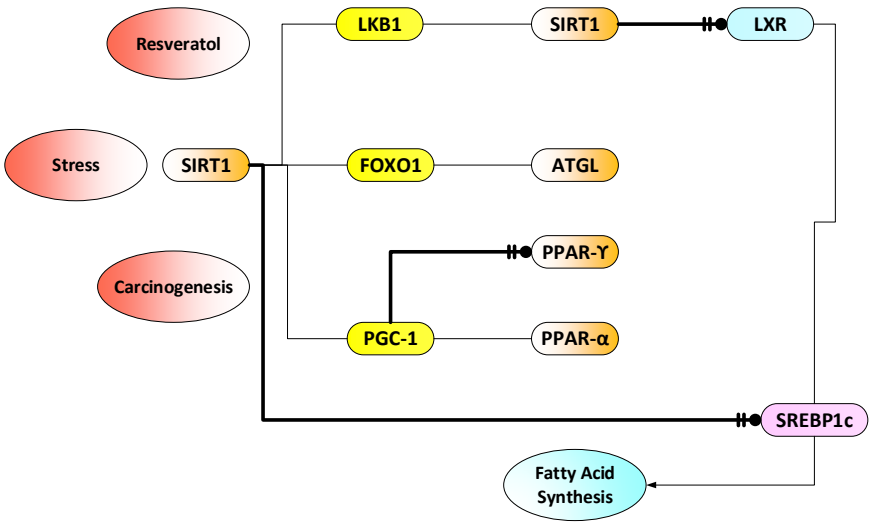
This indicates that SIRT1 does not require AROS as a physiological activator under all circumstances and leads to the conclusion that the positive role of AROS in regulating SIRT1 can respond to stimuli. As well as the variable suppression of p53 by AROS, this could have implications in the regulation of further SIRT1 targets, which may be regulated in a similar manner. It will be interesting to assess whether AROS is able to regulate multiple SIRT1 targets differently, suggesting that AROS has the capacity to act as a stimulus responsive orchestrator of SIRT1 activity. With SIRT1 implicated in diseases such as cancer, diabetes and

[5] See Kokkola et al, " *The modulation of protein deacetylase SIRT1 has a vast therapeutic potential in treatment of several aging-associated diseases. Active regulator of SIRT1 (AROS) is a small endogenous protein which was originally reported to activate SIRT1 through a direct interaction in cancer cells. We show that the interaction between the two proteins is weak and does not alter the activity of SIRT1 in noncancerous human cells. The results of different in vitro SIRT1 activity assays disclosed AROS as an inhibitor of SIRT1. The functional relationship between AROS and SIRT1 proved to be dependent on the biological context and experimental setting*."

neurodegeneration, greater understanding of its endogenous regulation could also lead to opportunities for therapeutic intervention.

Moreover Simmons et al have shown that SIRT1 when activated by carcinogenesis can impact FA synthesis and result in a complex of reactions in a positive feedback manner as shown below:

From: Simmons et al



Specifically Simmons et al note:

SIRT1, an NAD+-dependent deacetylase, has been described in the literature as a major player in the regulation of cellular stress responses. Its expression has been shown to be altered in cancer cells, and it targets both histone and non-histone proteins for deacetylation and thereby alters metabolic programs in response to diverse physiological stress. Interestingly, many of the metabolic pathways that are influenced by SIRT1 are also altered in tumor development.

Not only does SIRT1 have the potential to regulate oncogenic factors, it also orchestrates many aspects of metabolism and lipid regulation and recent reports are beginning to connect these areas. SIRT1 influences pathways that provide an alternative means of deriving energy (such as fatty acid oxidation and gluconeogenesis) when a cell encounters nutritive stress, and can therefore lead to altered lipid metabolism in various pathophysiological contexts. This review helps to show the various connections between SIRT1 and major pathways in cellular metabolism and the consequence of SIRT1 deregulation on carcinogenesis and lipid metabolism.

5.2 SOME OTHER GENES

It is worth examining a few other related genes. Consider first the relationship of SIRT1 to SOD2[6]:

SOD2 superoxide dismutase 2, mitochondrial: This gene is a member of the iron/manganese superoxide dismutase family. It encodes a mitochondrial protein that forms a homotetramer and binds one manganese ion per subunit. This protein binds to the superoxide byproducts of oxidative phosphorylation and converts them to hydrogen peroxide and diatomic oxygen.

Mutations in this gene have been associated with idiopathic cardiomyopathy (IDC), premature aging, sporadic motor neuron disease, and cancer. Alternate transcriptional splice variants, encoding different isoforms, have been characterized.

And for PARK2 we have[7]:

The precise function of this gene is unknown; however, the encoded protein is a component of a multiprotein E3 ubiquitin ligase complex that mediates the targeting of substrate proteins for proteasomal degradation. Mutations in this gene are known to cause Parkinson disease and autosomal recessive juvenile Parkinson disease. Alternative splicing of this gene produces multiple transcript variants encoding distinct isoforms. Additional splice variants of this gene have been described but currently lack transcript support.

From Powell et al we have as more detailed discussion of the functions of Sirt1:

The Sirtuin family of proteins (SIRT) encode a group of evolutionarily conserved, NAD-dependent histone deacetylases, involved in many biological pathways. SIRT1, the human homologue of the yeast Silent Information Regulator 2 (Sir2) gene, de-acetylates histones, p300, p53, and the androgen receptor. Autophagy is required for the degradation of damaged organelles and long-lived proteins, as well as for the development of glands such as the breast and prostate. Herein, homozygous deletion of the Sirt1 gene in mice resulted in prostatic intraepithelial neoplasia (PIN) associated with reduced autophagy.

Genome-wide gene expression analysis of Sirt1/ prostates demonstrated that endogenous Sirt1 repressed androgen responsive gene expression and induced autophagy in the prostate. Sirt1 induction of autophagy occurred at the level of autophagosome maturation and completion in cultured prostate cancer cells. These studies provide novel evidence for a checkpoint function of Sirt1 in the development of PIN and further highlight a role for SIRT1 as a tumor suppressor in the prostate.

The autophagy cleans up the cells and brings them back to a normal stasis. The recognition of Powell et al regarding the role of Sirt1 is key. They continue:

[6] http://www.ncbi.nlm.nih.gov/gene/6648

[7] http://www.ncbi.nlm.nih.gov/gene/5071

The role of SIRT1 in regulating prostate gland formation and androgen signaling in vivo was previously unknown. SIRT1is expressed in several cell types in the prostate gland including basal cells, luminal cells, and stromal cells. Given the evidence that SIRT1 functions as a tissue-specific regulator of cellular growth and that SIRT1 inhibits tumor cell line growth in nude mice, we sought to determine the role of endogenousSirt1 in regulating prostate gland development. Genome-wide expression profiling of Sirt1/ mice prostates and their littermate controls identified a molecular, genetic signature regulated by endogenous Sirt1.

The above clearly shows the understanding of the function of Sirt1. Note that the Powell work was in 2010 so that this understanding has been available for a while.

This signature highlights the ability of Sirt1 to inhibit androgen signaling and apoptosis in the prostate, while promoting autophagy. The Sirt1/ prostates demonstrated epithelial hyperplasia and PIN suggesting that Sirt1 promotes autophagy and inhibits prostate epithelial cell proliferation in vivo.

The above demonstrates the ability of Sirt1 to control androgen signalling. This also is a key factor in controlling prostate health.

Gene expression analysis further demonstrated that loss of endogenous Sirt1 inhibited autophagy. At a higher level of resolution, our studies demonstrated that SIRT1 antagonized DHT-mediated inhibition of autophagy in the prostate. Autophagy allows for degradation of proteins and organelles and is induced by nutrient withdrawal, rapamycin (inhibition of mTOR signaling), and hormone signaling.

Our findings are consistent with prior studies demonstrating that SIRT1 induces autophagy by deacetylating ATG5, ATG7, and ATG8 and inhibits AR signaling via deacetylation of the AR. Comparisons with previously published studies identified an overlap of 12.45% between genes regulated by endogenous Sirt1 and those targeted by androgens in the prostate gland and in prostate cancer cells. These results are consistent with prior findings that Sirt1 inhibits ligand-dependent AR signaling and gene expression in vitro

Again we come back to the role of autophagy. Perhaps the buildup of protein segments may act as normal cell blockage, inhibiting normal expression and control. The autophagy allows for a return to such normality. The emphasize this issue as follows:

The role of autophagy in cancer was proposed over 20 years ago. Autophagy appears to be essential for tumor suppression as well as for cell survival. Autophagy plays a prosurvival function for cancer cells during nutrient deprivation or when apoptotic pathways are compromised, a phenotype often accompanied by inflammation.

Again we see the putative role of inflammation. This appears to be a significant factor in PCa and the suppression of genes which deal with the remnants of inflammation seem to be a key benchmark in PCa progression. They continue:

In contrast, upon disruption of tumor suppressors, autophagy adopts a pro-death role with apoptotic pathways. In prostate, breast, ovarian, and lung cancer, loss of Beclin1 or inhibition of Beclin1 by the BCL-2 family of proteins causes defective autophagy, increased DNA damage, metabolic stress, and genomic instability.

These cancers also display neoplastic changes and increased cell proliferation, unlike cells overexpressing Beclin1, which undergo apoptosis. Loss of PTEN, p53, ATG4, ATG5, and MAP1LC31 (ATG8) are linked to tumorigenesis, whereas upregulation of PI3K, AKT, BCL-2, and mTOR are associated with inhibition of autophagy and the promotion of tumorigenesis.

Prostate cancer onset and progression are correlated strongly with aging and SIRT1 function governs aging in multiple species. Further studies will be required to determine whether this checkpoint function of Sirt1 in regard to prostate growth is linked to its role in organismal aging.

From Shackelford et al we have additional insights including pathway control issues as follows:

AMPK has recently been shown to increase sirtuin 1 (SIRT1) activity by increasing cellular NAD+ levels, resulting in the regulation of many downstream SIRT1 targets, including FOXO3 and peroxisome proliferator activated receptor-γ co-activator 1 (PgC1; also known as PPARgC1A), both of which have also been proposed to be direct substrates of AMPK46,76. As SIRT1 is also implicated in tumorigenesis, this connection between AMPK and SIRT1 might further explain how nutrients control cell growth. AMPK also suppresses mTOR-dependent transcriptional regulators to inhibit cell growth and tumorigenesis.

Two mTORC1-regulated transcription factors involved in cell growth are the sterol-regulatory element-binding protein 1 (SReBP1) and hypoxiainducible factor 1a (HIF1α). SReBP1 is a sterolsensing transcription factor that drives lipogenesis in many mammalian cell types. mTORC1 signalling is required for nuclear accumulation of SReBP1 and the induction of SReBP1 target genes78, and this can be inhibited by rapamycin or AMPK agonists

From Hines et al we have an expression of Sirt1 in terms of overall cell control:

The NAD + -dependent deacetylase SIRT1 is an evolutionarily conserved metabolic sensor of the Sirtuin family that mediates homeostatic responses to certain physiological stresses such as nutrient restriction. Previous reports have implicated fluctuations in intracellular NAD + concentrations as the principal regulator of SIRT1 activity. However, here we have identified a cAMP-induced phosphorylation of a highly conserved serine (S434) located in the SIRT1 catalytic domain that rapidly enhanced intrinsic deacetylase activity independently of changes in NAD + levels.

Attenuation of SIRT1 expression or the use of a nonphosphorylatable SIRT1 mutant pre- vented cAMP-mediated stimulation of fatty acid oxidation and gene expression linked to this path- way. Overexpression of SIRT1 in mice significantly potentiated the increases in fatty acid oxidation and energy expenditure caused by either pharmacological b -adrenergic agonism or cold exposure. These studies support a mechanism of Sirtuin enzymatic control through the

cAMP/PKA pathway with important implications for stress responses and maintenance of energy homeostasis

From Dominy et al we have:

From an evolutionary perspective, the nutrient-dependent control of protein acetylation through acetyltransferases and deacetylases is highly conserved and is a major mechanism for coupling metabolic activity with carbon/energy availability. The regulated acetylation of PGC-1a by GCN5 and Sirt1 is an excellent example: PGC-1a acetylation by GCN5 is favored under conditions of nutrient/energy abundance, whereas deacetylation by Sirt1 is favored under conditions of nutrient dearth and high energy demand

Finally Brooks and Gu state:

SIRT1 is a multifaceted, NAD+-dependent protein deacetylase that is involved in a wide variety of cellular processes from cancer to ageing. The function of SIRT1 in cancer is complex: SIRT1 has been shown to have oncogenic properties by down regulating p53 activity, but recent studies indicate that SIRT1 acts as a tumour suppressor in a mutated p53 background, raising intriguing questions regarding its mechanism of action.

Here we discuss the current understanding of how SIRT1 functions in light of recent discoveries and propose that the net outcome of the seemingly opposite oncogenic and tumour-suppressive effects of SIRT1 depends on the status of p53.

They clearly indicate the tumor suppressor role of Sirt1. p53 status is important but the observation above is truly intriguing if it is sustained.

5.3 MIRNA AND SIRT1

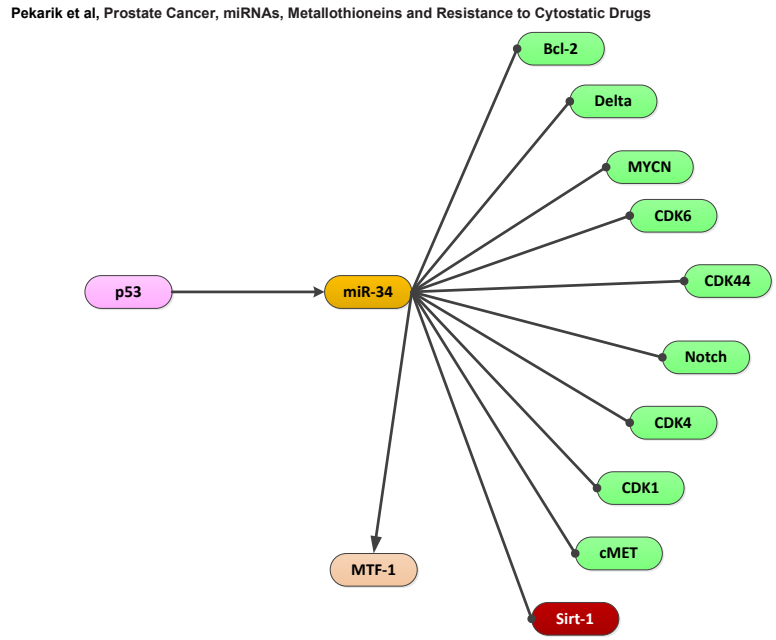
We have presented several results for SIRT1 and miRNA controls. We now expand this a bit more. The control of Sirt1 may be done via miRNAs. As Pekarik et al note:

Importance of miRNAs is underscored by the fact that nearly half of the genes coding miRNAs are located at fragile sites or at regions with lost homozygozity. For example, a loss of p-arm of chromosome 1 is a common finding in sporadic colon carcinomas. Among many genes associated with DNA repair, checkpoint functions, tumour suppressors, etc. are also multiple miRNAs.

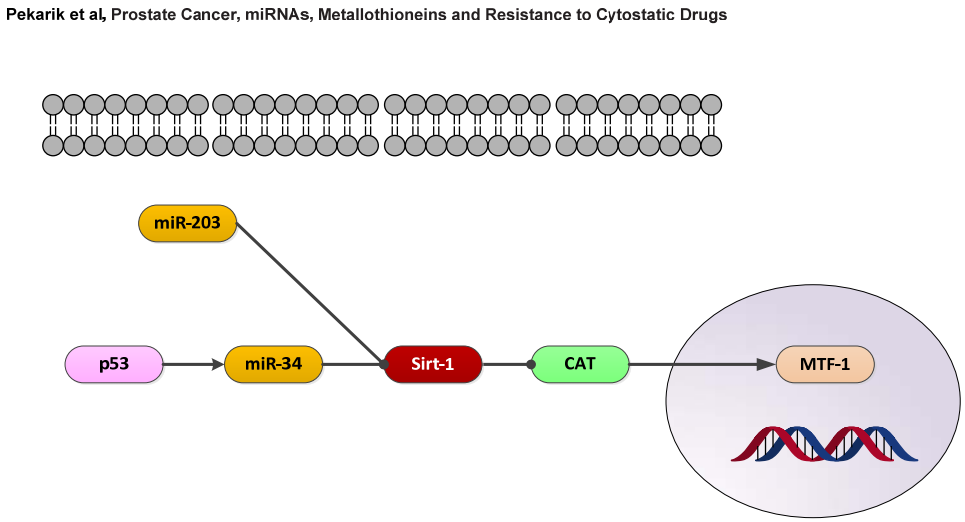
The most critical is miR-34a, directly regulated by tumour suppressor gene p53 and classified now as tumour suppressor itself. Ectopic miR-34a expression induces apoptosis and a cell cycle arrest in G1 phase. Downstream targets of miR-34 are Bcl2, MYCN, NOTCH1, Delta1, CDK4 and 6, Cyclin D1, Cyclin E2, c-Met, SIRT1, and E2F3, all the genes involved in apoptosis or proliferation and cell growth control…

We have discussed miRNAs and especially mrR-34 as part of PCa process. The control Sirt1 by miR-34 is a key observation It links back to a cause. Thus one may surmise that this is a potential

initiator and the miR-34 expression generated in some feedback manner with the inflammation which would have been controlled by Sirt1. We demonstrate that below.



And then we demonstrate the controlling process:



In addition miRNAs have also recently been shown to be facilitators of metastasis. There is a short review by Anastasiadou and Slack in Science which states:

Interestingly, exosomes contain messenger RNA (mRNA) and miRNA that can be transferred to other cells and regulate gene expression of the target cell. Likewise, miRNAs are present in

apoptotic bodies (small membrane vesicles that are produced by cells undergoing programmed cell death), or they are in the plasma, associated with Argonaute2 (AG02), the key effector protein of a miRNA-mediated gene silencing mechanism. However, miRNAs detected in human serum and saliva are mostly concentrated inside exosomes. Virally encoded miRNAs are also found in exosomes, indicating how oncogenic viruses could manipulate the tumor microenvironment. …

Melo et al. reveal a role of exosomes in cell-independent miRNA biogenesis that affects cancer progression. The authors show that only exosomes derived from cancer cells, but not those derived from normal cells, contain key enzymes involved in miRNA biogenesis such as Dicer, TAR (trans-activation response) RNA-binding protein (TRBP), and AGO2.

The exosomes also contain the membrane protein CD43, which plays a role in accumulating Dicer in cancer exosomes. The study also shows that Dicer-containing cancer exosomes process precursor miRNAs into mature miRNAs (including oncomiRs) over time, and upon encounter with normal human mammary epithelial, cells induces them to become cancerous.

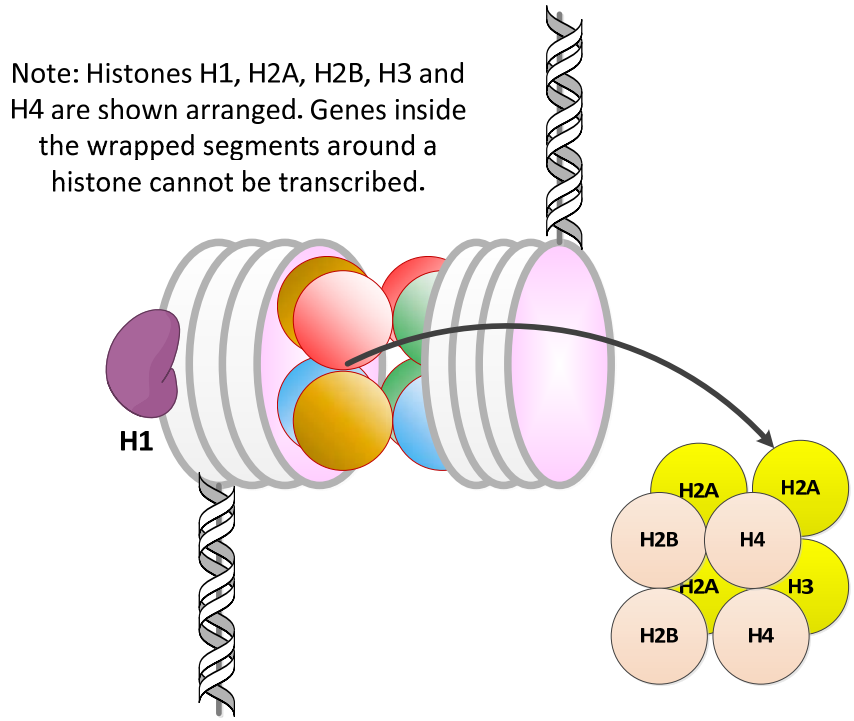
Thus, these epigenetic elements, the miRNAs, can spread throughout the body effecting changes in cells that are beyond fundamental intracellular effects. Thus the loss of Sirt1 expression may be the result of this exosomal effects.

5.4 METHYLATION AND ACETYLATION FACTORS

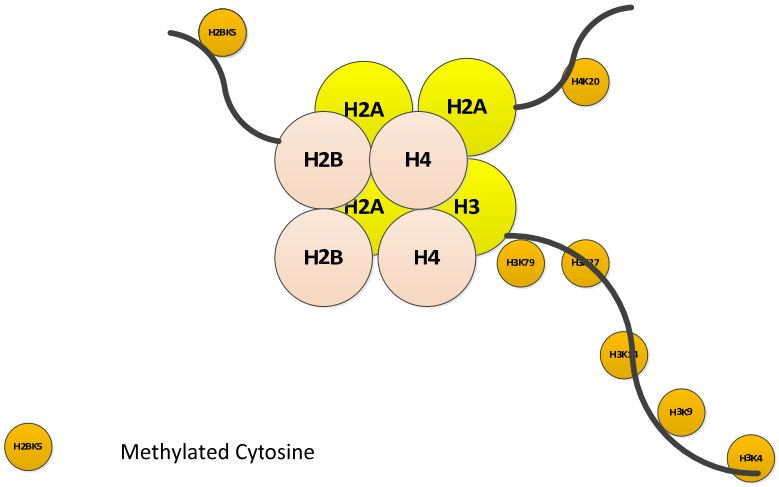
Methylation consists of the attachment of methyl groups on various elements of the genome. For our purposes we consider methylating the DNA on the CpG islands and methylation of the histones around which the DNA is wrapped. These effects have shown significant impact as well on PCa as well as many other cancers.

We have now described methylation, a rather simple process, and now we seek to discuss its influence on DNA. We start first at the top level of DNA, namely the chromosome. The DNA is often wrapped around histones, which are large protein masses that arrange themselves in a specific group. There are five main histones, H1, H2A, H2B, H3, and H4. They arrange themselves as shown below.

It appears as if one has eight large globes, each a histone, and they then allow the DNA to coil about them and in effect make certain that that specific segment of DNA is not read. Histones are another mechanism for DNA expression. They must be released so the DNA can be opened and then read in order for it to be expressed.

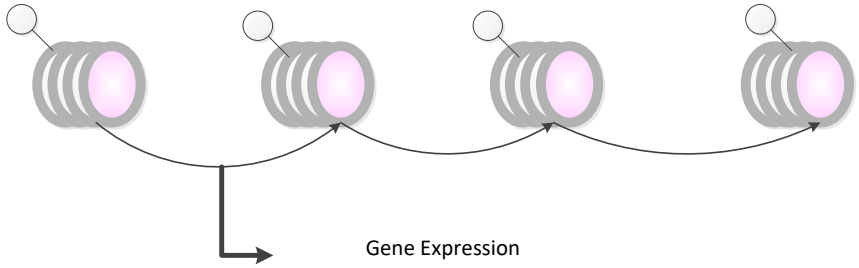


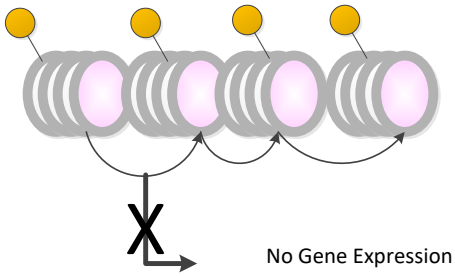
The specific arrangement of the histones is as shown below. It is not arbitrary but is a result of the specific surface charge arrangements on the histone proteins. We also depict the presence of methylated cytosines on this graphic, thus depicting the two major influences of methylation as well as acetylation, which we shall discuss.



Now what can happen is that the histone tails may become methylated, or acetylated, and when this occurs the histones may bind together or open up, depending on which lysine on the tail is affected. The open and close as a result of a methylation or acetylation is also called the histone

code. Methylate or acetylate the right ones and the DNA is curled and not expressible and do another set and the DNA can be expressed.



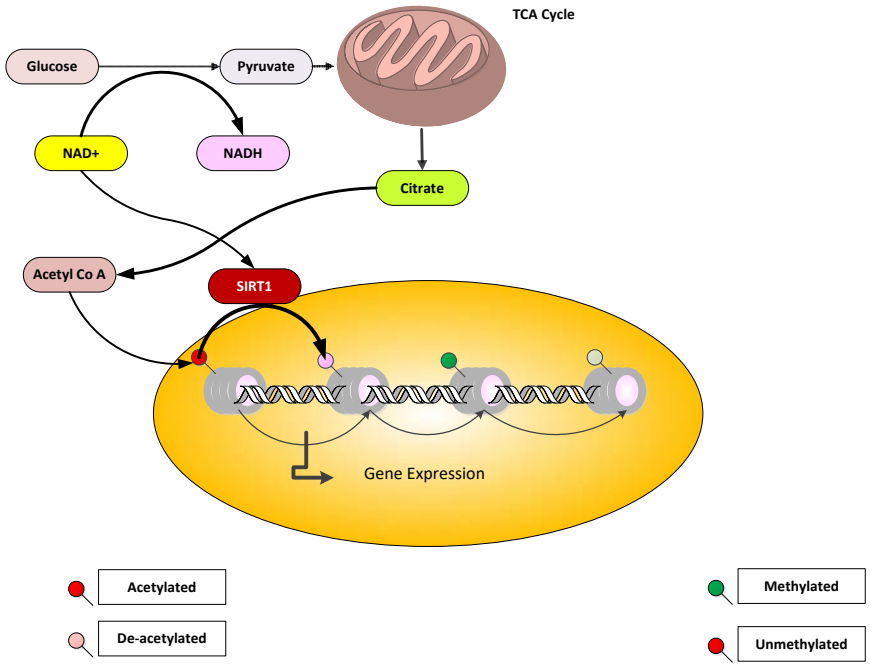


This Histone Code is shown below in the following Table.

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

Now we can use the above to understand the impact of these epigenetic factors via the interactions between Sirt1 and diet. In a recent paper by Labbe et al the authors examine dies and PCa. In particular they discuss the effect of Sirt1[8]. We show a modification of the Figure in the paper below. Glucose is converted to pyruvate via the action of NAD+ to NAH. Likewise this activates citrate to Acetyl-Co A and acetylates the histone changing its code but Sirt1 then deacytylates it to the ground state again. Thus loss of Sirt1 can potentially allow excess acetylated states which in turn does not allow the related genes to be expressed. Now from our discussions of miRNA exosomes we also understand that perhaps this down regulation of Sirt1 could be a result of metastatic spread of deregulating miRNAs. Although conjecture, the spread of miR34 via exosomes would result in suppression of Sirt1 as well as many other critical genes.

[8] http://www.nature.com/onc/journal/vaop/ncurrent/pdf/onc2014422a.pdf



The authors state as flows in their paper:

SIRT1 activity depends on the NAD+/NADH ratio modulated by glycolysis, while O-linked N-acetylglucosamine transferase uses GlcNAc produced by the hexosamine pathway. Pyruvate entering the tricarboxylic acid (TCA) cycle produces alpha-ketoglutarate, a critical cofactor for Jumonji domain-containing histone demethylase and TET. Acetyl-CoA is converted from the citrate generated by the TCA cycle and used as a donor by histone acetyltransferases.

Finally, the increase in ATP/ADP ratio from the TCA cycle also inactivates AMPK…. Under low-nutrient conditions, the NAD+/NADH ratio increases, activates SIRT1, which in turn de-acetylates and triggers ACECSs activity. Therefore, the pool of acetyl-CoA, which is governed by nutrient availability, controls the acetylation of metabolic enzymes as well as of histones at any given time.

As Melo et al state:

Exosomes are secreted by all cell types and contain proteins and nucleic acids. Here, we report that breast cancer associated exosomes contain microRNAs (miRNAs) associated with the RISC-Loading Complex (RLC) and display cell-independent capacity to process precursor microRNAs (pre-miRNAs) into mature miRNAs. Pre-miRNAs, along with Dicer, AGO2, and TRBP, are present in exosomes of cancer cells. CD43 mediates the accumulation of Dicer specifically in cancer exosomes.

Cancer exosomes mediate an efficient and rapid silencing of mRNAs to reprogram the target cell transcriptome. Exosomes derived from cells and sera of patients with breast cancer instigate

nontumorigenic epithelial cells to form tumors in a Dicer-dependent manner. These findings offer opportunities for the development of exosomes based biomarkers and therapies.

It would be expected that this may be found elsewhere, especially in PCa, since both PCa and Breast Cancer have great similarity[9].

Moreover, Braicu et al have presented a more comprehensive understanding of exosomes. Their observations are as follows:

Exosomes are key elements that facilitate intercellular communication; depending on their vesicular content ('cargo'), they can modulate tumor cells by influencing major cellular pathways such as apoptosis, cell differentiation, angiogenesis and metastasis. This communication can involve the exchange of molecules such as small noncoding RNAs (e.g. miRNAs) between malignant, non-transformed and stromal cells (in all directions). Exosomal miRNAs represent ideal candidates for biomarkers, with multiple applications in the management of an array of pathologies such as cancer. Manipulating exosomal miRNAs suggests new alternatives for patient-tailored individualized therapies.

They continue:

MiRNAs are short single-stranded (19–25 nucleotides in length) nonprotein-coding RNA transcripts (ncRNA) that are initially produced in the nucleus and then transported into the cytoplasm, where they undergo a series of steps to acquire maturation. Mature miRNAs regulate gene expression by binding (through watsonian complementarity) to the sequence of a target mRNA. This interaction results in translational repression and/or mRNA cleavage, which consequently decreases the levels of the mRNA coding protein. MiRNAs have been found to be aberrantly expressed in many diseases. For example, in cancer, the tumor microenvironment contains deregulated miRNA levels, and a reason for their altered levels is because they are being actively secreted as membrane-bound vesicular content.

Finally they state:

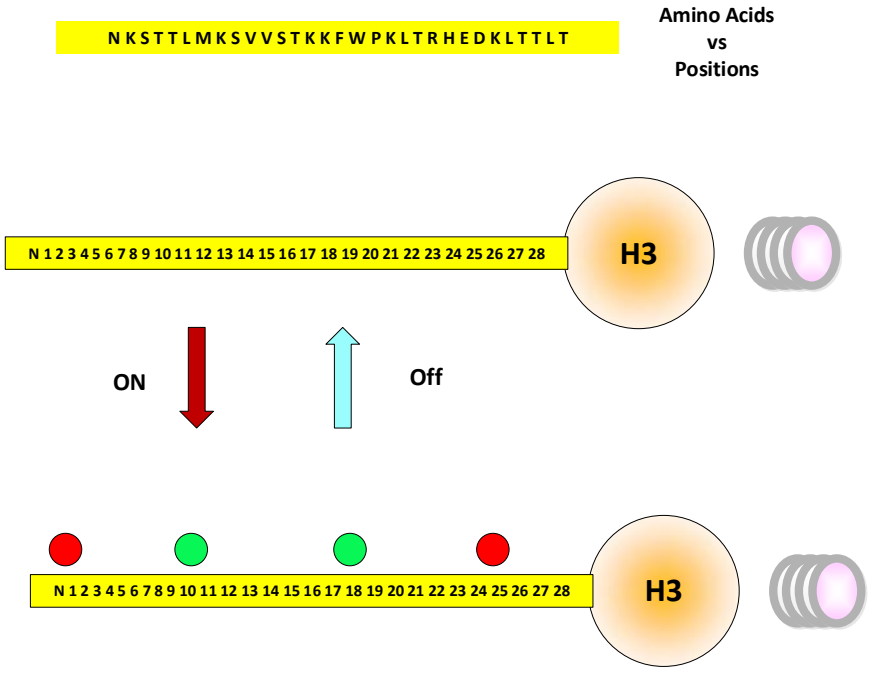
Immediately after their synthesis, exosomes are released and can remain in the extracellular space near the cell they originated from. Alternatively, they can also travel through body fluids such as blood, urine, amniotic fluid, saliva, lung surfactant, malignant effusions or breast milk. The end result of this dynamic process is a variety of regulative molecules being transported to different tissues in different places, and influencing cellular processes. Exosomes have been shown to carry proteins, many of which have the potential to influence multiple regulatory mechanisms. For example, exosomes can transport annexins that have the ability of altering the dynamics of the cytoskeleton.

Thus it is well understood that exosomes have not only the potential to allow one to see inside the cell, not only to transport to other cells but more importantly to act and a distributed means of control.

[9] See Telmarc White Paper 112 Prostate Cancer: miR-34, p53, MET and Methylation for detailed analysis.

5.5 THE HISTONE CODE

The Histone Code was described by Strahl and Allis in 2000 and it can be simply explained as follows. We examine it in a bit more detail here. It is a critical factor in opening and closing genes. First we layout below the relationship of a histone and its tail, the region for attachments.



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

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

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

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

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

1. Histone H3
2. K for lysine
3. Location 36 on the tail
4. methylated
5. tri methylated

Thus the notation can be specific as to the tuple:

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

As Jenuwein and Allis had noted in 2000:

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

From Tollefsbol we have:

Equally important in the fine tuning control of chromatin organization is the interplay between the histone modifications, DNA methylation and ATP-dependent chromatin remodeling. The large number of histone modifications and the possible interplay between them led to the proposition of the so-called "histone code hypothesis" in which "multiple histone modifications, acting in a combinatorial or sequential fashion on one or multiple histone tails, specify unique downstream functions". This hypothesis led the scientific community to adopt some metaphors to describe it such that the code is written by some enzymes ("writers"), removed by others ("erasers"), and is readily recognized by proteins ("readers") recruited to modifications through the binding of specific domains.

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

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

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

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

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

In summary we can articulate this as follows:

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

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

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

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

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

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

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

As Lu and Thompson have noted regarding the interaction between the histones and SIRT1 we have:

Histone acetylation—*Histone acetylation is catalyzed by HATs. Mammalian HATs are divided into five families which share a similar enzymatic reaction: HATs transfer the acetyl group of the acetyl-CoA to the lysine residues of histones and produce CoA as an end product. As demonstrated in yeast, elevated levels of acetyl-CoA can be sufficient to instruct cells to enter growth by promoting histone acetylation and expression of growth-related genes, suggesting that the availability of acetyl-CoA is a major metabolic input into histone acetylation. Indeed, it was shown that depending on the metabolic state, intracellular acetyl-CoA concentration shows a ~10-fold variation. Since the Km of most HATs is within the range the activities of HATs are likely sensitive to the fluctuation of intracellular acetyl-CoA levels.*

Histone deacetylation—*Enzymes that catalyze the removal of histone acetylation can be in principle divided into two groups based on structural and mechanistic similarities: classical HDACs and NAD+-dependent sirtuin family deacetylases. The deacetylation reaction is energetically favorable. Therefore sirtuins are intriguing as they catalyze the reaction in a seemingly wasteful way: one NAD+ molecule is hydrolyzed to yield nicotinamide and O-acetyl-ADP-ribose. The substrates of sirtuins are diverse and among seven members of the mammalian sirtuin family, SIRT1 and SIRT6 have been shown to localize to the nucleus and exhibit HDAC activities.*

DNA and histone methylation—*DNMTs and HMTs add methyl groups to DNA or lysine/arginine residues of histones, respectively. Although structurally diverse and possessing high substrate specificities, DNMTs and HMTs share a similar reaction mechanism: transferring a methyl group from S-adenosyl methionine (SAM) to the substrate with the formation of the by-product S-adenosyl homocysteine (SAH). SAM is derived from the essential amino acid methionine through methionine adenosyltransferase (MAT). It is possible to alter SAM levels through diets. However, SAH is a very potent inhibitor of DNMTs and HMTs and the key metabolic determinant of methyltransferase reactions is the rate of SAH clearance. SAH can be hydrolyzed to homocysteine. Homocysteine can be used to regenerate methionine, a step catalyzed by methionine synthetase and dependent on one-carbon metabolism. Alternately, homocysteine can enter the transulfuration pathway to generate cysteine, the precursor for glutathione synthesis.*

DNA and histone demethylation—*A covalent methyl group is chemically stable. Therefore DNA and histone methylation were considered as relatively static epigenetic marks. However during embryonic development there is extensive remodeling of the cellular methylome, suggesting the existence of enzymes that actively remove methylation marks. Indeed in recent years, a variety of HDMs and DNHDs have been identified. The first identified HDM is LSD1. The histone demethylation reaction catalyzed by LSD1 involves the reduction of co-factor flavin adenine dinucleotide (FAD) to FADH2 and the release of formaldehyde as a by-product. As recycling of FAD requires converting molecular oxygen to hydrogen peroxide*

6 DYNAMICS

We have examined the dynamics of cancer progression under the construct of the classic model. Namely under the assumption that intravasation and extravasation is of the malignant cell itself. A neutrophil is about 10 μm in diameter and exosomes are roughly the same size. Kang et al note the size of a prostate cell is about 30 μm[10]. Neutrophils manages to stick and pass through the vasculature. One suspects that a similar exosome could do likewise.

In our 2013 paper on Cancer Cellular Dynamics we developed a model for the average density of a cancer cell of a specific type at a location x and time t as follows:

$$\frac{\partial \overline{n_k(x,t)}}{\partial t} = L_k \overline{n_k(x,t)} - \lambda_{k,k} \overline{n_k(x,t)} + \sum_{j=1; j\neq k}^{N} \lambda_{k,j} \overline{n_j(x,t)}$$

Thus the result for the average is a set of linked partial differential equations. Note we have modified the L operator to reflect specificity for k. The added terms reflect the movement of cell types from one class to another.

This is a powerful equation. It tells us how specific cells diffuse, flow and reproduce, and then how they migrate to new types of cells.

Let us take it one step further. Recall:

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

define

$$\widetilde{L_k} = L_k - \lambda_{k,k}$$

Now consider a vector of all n possibilities and we can determine the average vector of these as follows:

$$n(x,t) = \begin{bmatrix} n_1(x,t) \\ \dots \\ n_N(x,t) \end{bmatrix}$$

And where the average of the vector is the average of the above. Then we readily have the equation for all the average n as follows:

[10] *As target cells in this research, the average diameters of the RWPE-1 and PC-3 cells were 29.48 μm and 32.39 μm, respectively, which were estimated from microscopic images (standard deviation of the RWPE-1 and PC-3 were 6.94 μm and 7.00 μm, respectively).*

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

Where:

$$\tilde{L} = \left[\tilde{L}_1 ..... \widetilde{L_N} \right]$$

and

$$\Lambda = \begin{pmatrix} -\lambda_{11} & \lambda_{12} & \lambda_{13} \\ \lambda_{21} & -\lambda_{22} & \lambda_{23} \\ \lambda_{31} & \lambda_{32} & -\lambda_{33} \end{pmatrix}$$

where the L are spatial characteristic we had developed and are measurable and the second matrix Λ is the transition rates for mutation changes.

The above model can be experimentally verified but it depicts the Classical approach. It is a spatio-temporal stochastic model where the sole driving factor is the movement and change and propagation of cells. Now we can readily use this same approach to do the same for the EV Neo-Classical model. This will be examined at a later date.

7 THERAPEUTICS

The question of targeting miRNAs for therapeutic purposes when transmitted via exosomes is a growing field. The recent book by Mansoor and Ramesh is an excellent example of much of the work being performed in this area. We briefly touch on some of these issues as follows.

As Vader et al note:

Intercellular communication is fundamental to survival and maintenance of homeostasis in all multicellular systems. By contrast, dysregulated pathways of communication appear to drive cancer development and progression. The development of successful anticancer treatments will therefore crucially depend on increasing our understanding of the complexity of interactions between tumour cells and other cells.

Communication between cells takes place via direct cell-to-cell contact, for example, through adhesion molecules, gap junctions, and nanotubes, or via soluble communication signals such as cytokines, growth factors, and hormones secreted by both tumour and nontumour cells. However, an additional novel mechanism that can operate over both short and long distances has recently emerged, based on the release and uptake of membrane-bound vesicles termed extracellular vesicles (EVs).

The recent discovery that EVs are able to convey complex multimolecular biological messages between cells has the potential to revolutionise our understanding of the communication circuitry in cancer. Further, EV research is anticipated to directly advance various areas of clinical cancer science, including cancer diagnostics and therapy. Biogenesis, composition, and function of extracellular vesicles.

Over the past decade, research efforts into EV biology, function, and application have dramatically increased. It has now become clear that virtually all cell types release EVs, constitutively and/or upon activation (e.g., as a result of hypoxia or shear stress). EVs have been traditionally classified based on their cell or tissue of origin, for example, prostasomes are derived from prostate cells, and oncosomes are derived from tumour cells. More recently, however, different classifications of EVs are being used, based on intracellular origin or biogenesis mechanism. Using this approach, although there is currently little consensus in the field regarding nomenclature due to differences in classification criteria, three main classes of EVs can be distinguished: exosomes, microvesicles (also referred to as ectosomes or microparticles), and apoptotic bodies.

Exosomes have been defined as originating from multivesicular bodies (MVBs) and are secreted upon fusion of MVBs with the plasma membrane. Exosomes are believed to range between 40 and 150 nm in size with a buoyant density of 1.13–1.19 g/cm3, and are often characterised using marker proteins such as ALG-2-interacting protein X (ALIX) and tumour susceptibility gene 101 (TSG101), which indicate an endocytic origin

There are many other recent studies examining this possibility. The work of Tai et al note:

Cargoes of tumor-derived exosomes attribute to cancer development. Thus, alternative therapeutic strategies, such as blockage of exosome production, secretion, and exosome-mediated cell-cell communication, as well as ablation of specific active exosomal cargos, have been proposed as novel cancer interventions.

Indeed, inhibition of the ESCRT-dependent or the ESCRT-independent mechanism-mediated exosome biogenesis, such as syndecan/syntenin/ALIX signaling or sphingomyelinases, respectively, has shown detrimental effects in exosome production and on cancer progression. Another critical protein significant in exosome secretion, Rab27 small GTPase, is involved in regulating the docking of multivesicular endosomes onto the plasma membrane and the size of multivesicular endosomes.

Cancer proliferation and metastasis were hindered upon inhibiting Rab27a. An inhibitor of clathrin-mediated endocytosis, chlorpromazine, was shown to impede cancer malignancy in vitro by targeting the mechanism of exosome uptake by endocytosis or macropinocytosis. Furthermore, the surface proteins of tumor-derived exosomes display specific glycosylation patterns that are involved in the regulation of exosome uptake by recipient cells. Such a finding suggests that alteration in the glycosylation of exosomal proteins can be potent in cancer progression.

Recently, a cancer treatment strategy for extracorporeal hemofiltration of exosomes from the circulation by an affinity plasmapheresis platform has been proposed, suggesting that removal of exosome from the circulatory system provides an additional strategy for therapeutic reagents to block the oncogenic signal on cancers. Together, these studies suggest that various potential therapeutic strategies by intercepting biogenesis, secretion, or uptake of tumor-derived exosomes are promising means for the development of anticancer therapies.

Similarly we have Sullivan et al who have reported:

Given the growing evidence of EV involvement in cancer pathogenesis, it seems intuitive to explore translational approaches that lead to their inhibition. Current studies are utilizing different techniques to inhibit vesicle formation, release, and cell uptake as well as blocking specific components of the EV.

The drug amiloride has been shown in vivo to block secretion of tumor-derived EVs that contain membrane-associated heat shock protein 72 (HSP72) . HSP72 is constitutively expressed in many cancers and is associated with a poor prognosis . Furthermore, amiloride was shown to inhibit ceramide, an important mediator of EV biogenesis. Another drug, diannexin, inhibits phosphatidylserine, a regulator of cell adhesion, and EV endocytosis . Rab27 is a protein demonstrated to have a significant role in EV secretion .

In highly metastatic mouse models of both melanoma and breast cancer, knocking down Rab27 led to a significant reduction of tumor EV production, primary tumor size, and metastasis However, since EVs are also essential participants in normal cell physiology, better techniques are required to distinguish and target pathological versus physiological EVs. As mentioned

previously, EVs have been used as cancer vaccines by carrying and providing tumor-specific antigens to immune cells, which prime the immune system and create a powerful immunological response against the tumor.

Some of the first phase I clinical trials applying this methodology took place in melanoma and non-small-cell lung carcinoma where dendritic cell-derived EVs were loaded with MHC/tumor antigen and delivered back to patients. These studies demonstrated that cancer vaccines are both feasible in creation and safe for administration. Later, EVs from the malignant ascites of colorectal cancer patients were isolated, mixed with specific cytokines, and administered back to the patient as a subcutaneous immunization.

The investigators reported that combining ascites EVs with granulocyte-macrophage colony-stimulating factor induced specific antitumor cytotoxic T-lymphocyte activation . Furthermore, in a phase II clinical trial including patients with advanced small-cell-lung carcinoma, the administration of dendritic cell-derived EVs caused an increase in natural killer cell activity and longer progression-free survival for patients with low initial expression of natural cytotoxicity receptor NKp30 .

The realization that EVs are efficient vehicles for cell-to-cell communication has subsequently given rise to investigations of their use as a method for drug delivery. EVs display many potential advantages over current approaches. They are stable in serum, have specific cell-targeting capabilities, can overcome natural barriers such as the immune system or the blood–brain barrier, and can deliver molecules such as miRNAs or siRNAs that are readily degraded in the serum . Multiple methods have been reported to successfully load EVs with a desired drug. Hydrophobic drugs have been demonstrated to integrate with EVs successfully by simply mixing and allowing the drug to pass through the EV lipid bilayer membrane .

The loading of hydrophilic drugs has proven to be more challenging, but still possible by methods including electroporation, sonication, saponin-mediated permeabilization, and freeze–thaw cycles . Perhaps the most challenging aspect of EV-mediated drug deliver is the efficient targeting of specific cell types. Some groups have used transfection-based approaches to encourage cells to express organ-specific ligands or receptors that are loaded into EVs, released from the cell, and then isolated and collected for successive drug loading.

Other groups are experimenting with iron oxide nanoparticles in combination with a drug within EVs to target specific areas of the body by the application of a magnetic field gradient .

Tai et al further note regarding the use of EV for drug delivery:

Resembling liposomes, naturally secreted exosome vesicles have garnered much attention as drug-delivery vehicles. First of all, the nanometric-sized exosomes can be easily transferred between cells. Second, the lipid bilayer-membrane structure of exosomes confers a protected environment for bioactive molecules from degradation in the extracellular milieu. Third, exosomes show lower immunogenicity and toxicity than other drug-delivery strategies. Last,

exosomes bearing specific surface proteins, such as integrins, can direct themselves to specific organs. These features of exosomes implicate that exosomes can be efficient drug-delivery vesicles for the delivery of anticancer agents, siRNAs, or proteins.

Interestingly, exosomes transfer anticancer drugs through the BBB, leading to cytotoxic effects in Danio rerio brain cancers. Prevalently, exosomes loaded with anticancer drug derived from autologous cancers can be taken up by parental cancer cells through endocytosis, leading to increased cytotoxicity in parental cancer cells.

In terms of targeting specificity, av integrin-specific RGD (Arg-Gly-Asp) peptide was fused on exosomes loaded with anticancer drug (ie, doxorubicin) to significantly improve exosome uptake by av integrin-positive cancer cells, leading to inhibition of cancer growth. Intrinsically, exosomes have been recognized as novel cell-free vaccines in immunotherapy.

Cancer antigens loaded into exosomes derived from autologous dendritic cells facilitate anticancer immune responses (ie, induced natural killer, NK, cell effector functions) in patients with advanced non-small-cell lung cancer. Further study used exosomes from interferon-y-mature dendritic cells to accelerate anticancer immune responses in both NK and T cells.

Increase in NK cell activity and longer progression-free survival rate were observed in patients with advanced non-small-cell lung cancer. Together, these studies suggested that exosomes function as potential drug-delivery vehicles or cell-free vaccines in anticancer therapies.

To be effective, however, the therapeutics must address the specific miRNAs, the details of the exosome transport, the receptors facilitating the entry into the distant cell and many other factors yet to be examined.

8 IMMUNOTHERAPY

Immunotherapy for cancers has become a highly productive field. The development of various checkpoint inhibitors as well as CAR-T cells use the knowledge of cancer cell markers to target and attack the malignant cells. The question we would pose is:

What can be accomplished targeting the exosome and its related miRNA as both an early stage immunotherapeutic as well as a later stage one?

Work in this area is progressing but the focus is on exosomes as targets.

As Maas et al note:

EVs play a complex role in immune responses and can influence both adaptive and innate immunity through exchange of EVs among multiple types of immune cells. Intracellular vesicle transport is crucial for MHC class-II mediated antigen presentation. After delivery to the plasma membrane from the Golgi, clathrin-mediated endocytosis of cellular membrane MHC class II complexes can result in the incorporation of MHC class-II αβ dimers into ILVs within MVBs and their limiting membrane.

Antigen peptide binding to MHCII occurs within this compartment. Peptide-bound MHCII in the limiting membrane of the MVB can be directly recycled back to the cellular membrane. In addition, some MVBs directly fuse with the plasma membrane releasing the ILVs with incorporated peptide-MHCII into the extracellular space as EVs.

These EVs are potent in inducing an immune response, as antigen-specific T-cell activation can be induced by dendritic cell (DC) secreted-EVs, either indirectly through DCs that express the co-stimulatory molecules CD80 (B7-1) and CD86 (B7-2) or directly by ICAM-1-presenting mature DC-derived EVs. EVs purified from DCs have been shown to differentiate T helper cells towards a T-helper 1 (Th1) phenotype and to enhance in vivo immunogenicity.

Much of the EV exchange between T cells and antigen presenting cells (APCs) takes place at the immune synapse, a site of cell-cell adhesion that promotes T-cell activation. In addition to the EV flow from APCs to T-cells for the purpose of antigen presentation, T-cells also release EVs at the immune synapse in order to influence target cells. T-cells have been shown to release both exosomes and microvesicles. In the case of exosomes, T cell MVBs were shown to translocate to the immunological synapse before fusion with the plasma membrane. These exosomes contain miRNAs (e.g miR-335) that are internalized into the APCs and subsequently reduce target mRNA expression levels, as detected in a miR-335 target SOX4-luciferase reporter assay.

In addition, through a combination of live cell imaging, correlative light-electron microscopy and biochemical experiments, T cell receptor (TCR)-containing EVs were found to bud from T cells at the immune synapse and engage peptide-bound MHC in recipient antigen-presenting B-cells. These experiments suggest that EVs are part of a dynamic interchange between immune cells that includes communication taking place at a distance, such as stimulation of transmembrane receptors.

Other ways to use the exosomes for delivery have been considered. Zhang et al note:

Dendritic cell-derived exosomes (dexosomes) have been developed as immunotherapeutic anticancer agents. Tumor peptide-pulsed DC-derived exosomes suppress growth of established murine tumors in a T cell dependent manner. Exosomes secreted by living tumor cells contain and transfer tumor antigens to dendritic cells and induce potent CD8+ T cell-dependent antitumor effects on mouse tumors. Dexosomes have entered clinical trials for colorectal cancer, metastatic melanoma, and non-small cell lung cancer and have achieved modest therapeutic effects.

This is just the beginning of the focus in this area. Exosomes can be used to transport and effect therapeutic actions, but as we have argued herein, they may be the target themselves.

9 DIAGNOSTICS AND PROGNOSTICS

Understanding the use of exosomes as diagnostic and prognostic tools is the other dimension from that of a therapeutic. It is now possible to collect and examine exosomes and their contents and then to assess what they imply. One should remember that there is still the "needle in the haystack" issue since there are very few exosomes in circulation. But the ones there do reflect the status of a possible malignancy. A great deal of work profiling these must still be done.

9.1 INITIATORS

We first present some summaries in miRNA initiators as well as other exosome elements. From Tai et al we have the following Table of putative initiators.

Exosomal bioactive molecules	*Type of bioactive molecule*	*Mechanism*	*Functional effect*	*Process*	*Cancer type*
Delta-like 4	Protein	Inhibit Notch signal	Increase vessel branching and length	Modification of cancers and tumor microenvironment	
EGFR vIII	Protein	Activate AKT and MAPK signal	Increase anchorage-independent growth		Glioma
Integrins	Protein	Activate Src and upregulate proinflammatory S100 genes	Direct exosomes to specific tissues	Metastatic organotropism	Breast cancer
MET	Protein	Activate MET signal	Increase prometastatic activity of bone marrow cells	Priming premetastatic niches	Melanoma
MIF	Protein	Activate TGF-P signal-induced fibronectin production	Increase liver premetastatic niche formation	Increase liver metastatic burden	Pancreatic cancer
TGF-P	Protein	Activate SMAD-related signal	Increase fibroblast FGF2 production	Trigger fibroblast to myofibroblast differentiation	
TGF-P	Protein		Increase mesenchymal stem cell differentiation into myofibroblasts	Increase cancer proliferation and invasiveness	Prostate cancer
TGF-P1	Protein	Activate antiapoptotic and pro-survival signals	Increase proliferation and survival	ncrease cancer growth	Chronic myeloid leukemia
Tspan8	Protein		Increase endothelial cell proliferation, migration, and sprouting	Increase angiogenesis	Adenocarcinoma

Exosomal bioactive molecules	*Type of bioactive molecule*	*Mechanism*	*Functional effect*	*Process*	*Cancer type*
Snail and miR-146a	**Protein and miRNA**		**Increase proliferation and drug resistance**	**Increase cancer proliferation and survival**	**Pancreatic cancer**
miR-9	**miRNA**		**Increase CAF-like property**	**Increase cancer growth**	**Breast cancer**
miR-17-92 cluster	**miRNA**		**Increase endothelial cell migration and tube formation**	**Increase angiogenesis**	**Leukemia**
miR-21	**miRNA**	**Regulate PTEN/PI3K/AKT signal**	**Inhibit apoptosis**	**Increase drug resistance**	**Gastric cancer**
miR-105	**miRNA**	**Downregulate tight junctions (ZO-1)**	**Destroy vascular endothelial barrier**	**Increase metastasis**	**Breast cancer**
miR-181c	**miRNA**	**Downregulate PDPK1/cofilin signal**	**Destroy blood-brain barrier**	**Increase brain metastasis**	**Breast cancer**
miR-200	**miRNA**	**Regulate gene expression and EMT**	**Increase cancer colonization in the lung**	**Increase metastasis**	**Breast cancer**
miR-222-3p	**miRNA**	**Regulate SOCS3/STAT3 pathway**	**Increase TAM polarization**	**Increase cancer progression**	**Epithelial ovarian cancer**
ZFAS1	lncRNA	Regulate MAPK signal and EMT transcription factors	Increase cell cycle progression and EMT	Increase cancer growth and metastasis	Gastric cancer
hTERT mRNA	mRNA		Transform nonmalignant fibroblasts into telomerase-positive	Modification of cancer microenvironment	

An additional target Table is presented by Zhang et al:

Exosomal cargo	*Secreting cell*	*Recipient cell*	*Function*
EGFRvIII	Glioblastoma cells	Glioblastoma cells	Promotes tumor cell growth
Angiogenin, IL-8, VEGF	Glioblastoma cells	Endothelial cells	Promotes tube formation
ΔNp73	Colon cancer cells	Colon cancer cells	Promotes tumor cell proliferation and therapy resistance
KRAS	Colon cancer cells (mutant KRAS)	Colon cancer cells (wild-type KRAS)	Enhances tumor cell growth
MET	Melanoma cells (highly metastatic)	Bone marrow progenitor cells	Promotes tumor growth and metastasis
HIF-1α	Nasopharyngeal carcinoma (NPC) cells (EBV-positive)	NPC cells (EBV-negative)	Promotes tumor cell migration and invasion
αvβ6 Integrin	Prostate cancer cells	Prostate cancer cells	Promotes tumor cell migration
Survivin	Cervical cancer cells	Cervical cancer cells	Inhibits genotoxic stress-induced apoptosis and promotes cell proliferation
Wnt5a	Macrophages	Breast cancer cells	Enhances tumor cell invasion

Exosomal cargo	*Secreting cell*	*Recipient cell*	*Function*
Wnt3a	Diffuse large B-cell lymphoma side population (SP) cells	Neighboring non-SP cells	Modulates SP–non-SP transition and promotes tumor progression
FasL	Activated CD8+ T cells	Melanoma cells, lung cancer cells	Induces MMP9 expression and promotes lung metastasis
IL-6, CCL2, fibronectin	Multiple myeloma (MM) BM-MSCs	MM cells	Promotes tumor cell growth
Hsp72	Murine thymoma, mammary carcinoma, colon carcinoma cells	MDSCs	Induces immunosuppression and enhances tumor growth
TF	Squamous cells, colon cancer cells	Endothelial cells	Promotes coagulation
CD39, CD73	Bladder, colorectal, prostate, breast cancer cells	T cells	Induces adenosine production and inhibits T cell activation
TGF-β	Mesothelioma, prostate, bladder, colorectal, breast cancer cells	Fibroblasts	Induces myofibroblast differentiation and promotes tumor angiogenesis and growth
TGF-β	Prostate cancer, gastric cancer	MSCs	Induces myofibroblast differentiation and promotes angiogenesis and invasiveness
TGF-β	Pleural effusions of mesothelioma patients	NK cells, CD8+ T cells	Downregulates NKG2D expression and impairs cell killing activity
MICA*008	Cervical cancer cells	NK cells	Decreases NKG2D expression and reduces NK cytotoxicity
TGF-β, PGE2	Murine mammary adenocarcinoma cells	Bone marrow myeloid cells (CD11b+Ly6G+)	Induces MDSCs accumulation and immunosuppression
CCL20	Nasopharyngeal carcinoma cells	Regulatory T cells	Recruits and induces Treg conversion
KIT	Mast cells	Lung cancer cells	Accelerates cell proliferation
KIT	Gastrointestinal stromal tumor (GIST) cells	Progenitor smooth muscle cells	Increases tumor invasiveness
Wnt11	Fibroblasts	Breast cancer cells	Promotes tumor metastasis
MIF	Pancreatic cancer cells	Liver Kupffer cells	Promotes metastasis
Hsp70	Renal cancer cells (murine Renca cell line)	MDSCs	Induces MDSCs activation and enhances tumor growth
Adrenomedullin	Pancreatic cancer cells	Adipocytes	Promotes lipolysis
S1P, CCL20, PGE2	Enteropathogenic bacteria-stimulated intestinal epithelial cells	Th17 cells	Promotes the development of colon cancer
miR-9	Lung cancer, melanoma, pancreatic cancer, glioblastoma, colorectal cancer cells	Endothelial cells	Induces tumor angiogenesis

9.2 TARGETS

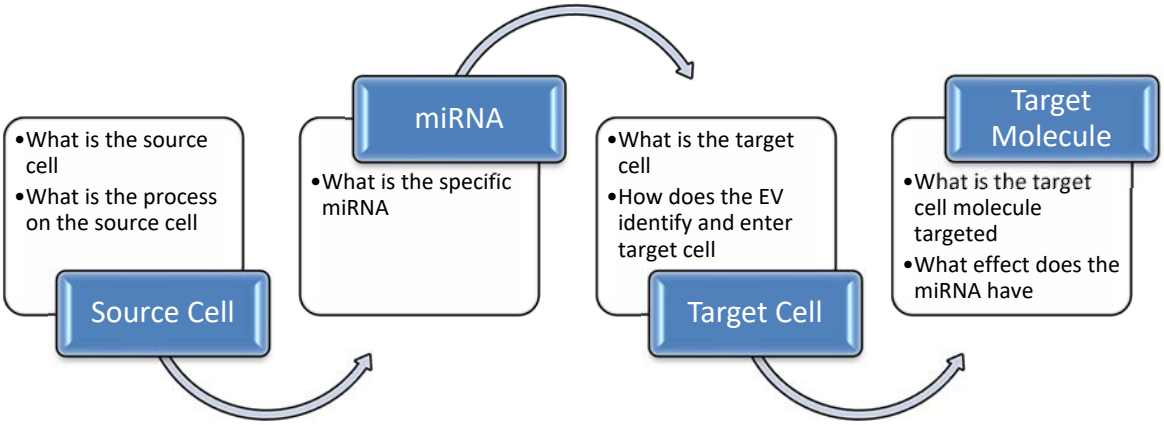
Targets are different than those above. Targets allegedly tell one what the cancer is and possibly where and they may become therapeutic targets as well. Zhang et al continue by presenting the following list:

Exosomal cargo	*Secreting cell*	*Recipient cell*	*Function*
miR-125b, 130b, 155	Prostate cancer (PC) cells	PC patient adipose-derived stem cells (pASCs)	Induces neoplastic transformation
miR-135b	Multiple myeloma cells (under chronic hypoxia condition)	Endothelial cells	Enhances endothelial tube formation
miR-10b	Metastatic breast cancer cells	Mammary epithelial cells	Promotes cell migration

Exosomal cargo	*Secreting cell*	*Recipient cell*	*Function*
miR-92a	Chronic myeloid leukemia (CML) cells	Endothelial cells	Promotes cell migration and tube formation
miR-210	CML cells (under hypoxia condition)	Endothelial cells	Promotes angiogenic activity
miR-223	IL-4-activated macrophages	Breast cancer cells	Promotes cell invasion
miR-222	Drug-resistant breast cancer cells	Drug-sensitive breast cancer cells	Transmits chemoresistance
miR-584, 517c, 378	Hepatocellular carcinoma (HCC) cells	HCC cells	Promotes HCC cell growth and metastasis
miR-21, 29a	Lung cancer cells	Macrophages	Promotes tumor metastasis
miR-105	Metastatic breast cancer cells	Endothelial cells	Destroys tight junction, induces vascular permeability, and promotes metastasis
Pre-miRNAs, RISC- loading complex	Breast cancer cells	Non-tumorigenic epithelial cells	Induces cell transformation
miR-24-3p, 891a, 106a-5p, 20a-5p, 1908	Nasopharyngeal carcinoma	T cells	Promotes T cell dysfunction and tumor progression
miR-221, 222	Gastric cancer tissue derived MSCs	Gastric cancer cells	Enhances tumor cell migration
miR-122	Breast cancer cells	Lung fibroblasts, brain astrocytes, and neurons	Reprograms systemic energy metabolism and facilitates metastasis
miR-23b	Bladder cancer cells (cellular disposal by exosome release)	None	Acquires metastatic potential
miR-503	Endothelial cells	Breast cancer cells	Impairs tumor cell growth
miR-140	Preadipocytes	Ductal carcinoma in situ (DCIS) cells	Enhances tumorigenesis
miR-127, 197, 222, 223	Bone marrow stromal cells	Breast cancer cells	Decreases cell proliferation and induces cell quiescence
TUC339	Hepatocellular carcinoma (HCC) cells	HCC cells	Promotes tumor cell growth and inhibits cell adhesion
Linc-ROR	HCC cells	HCC cells	Reduces chemotherapy sensitivity

The above is a powerful presentation. It demonstrates the miRNA, the secreting cell, the target cell and the action. What is seems to miss is the gene or other target which the miRNA has effects on.

What would be highly useful would be a mapping as follows:



Addressing the above issue would be essential. This would be the first step in diagnostics and prognostics as well as therapeutics.

Zhang et al lay forth the following specific targets which have been considered in the related cancers:

Exosome Cargo	***Cancer Type***
CD34	Acute myeloid leukemia
EDIL-3/Del1	Bladder cancer
miR-101, 372, 373 Breast cancer	Breast Cancer
miR-21, 146a	Cervical cancer
Let-7a, miR-1229, 1246, 150, 21, 223, 23a	Colon cancer
CD147, CD9	Colon cancer
miR-21	Esophageal squamous cell carcinoma
LINC00152	Gastric cancer
miR-718	Hepatocellular carcinoma
miR-17-3p, 21, 106a, 146, 155, 191, 192, 203, 205, 210, 212, 214	Lung cancer
LRG1	Lung cancer
TYRP2, VLA-4, Hsp70, MET	Melanoma

Exosome Cargo	*Cancer Type*
CD63, caveolin-1	Melanoma
Galectin-9	Nasopharyngeal carcinoma
Claudin-4	Ovarian cancer
miR-21, 141, 200a, 200b, 200c, 203, 205, 214 miR-1246, 4644, 3976, 4306	Ovarian cancer
PTEN	Prostate cancer
Survivin	Prostate cancer
PSA, PSMA	Prostate cancer
miR-1290, miR-375	Prostate cancer
LncRNA-p21	Prostate cancer

There clearly are a significant number of miRNAs but also a large collection of alternatives as well. Some are results and some clearly are initiators. Separating them and understanding their functions is critical but time consuming.

10 OBSERVATIONS

We present several concluding observations as to the above materials.

10.1 PARADIGM CHANGES

The paradigm change we propose herein may have some merit as has been observed by others. Yet the statement as bold as made herein does not seem to be in the current literature. The problem is that the details are yet to be obtained and many exosomes transport no active materials and thus may just be noise. The targeting of miRNA is supposed based upon its unique capability of gene expression modification.

10.2 DIAGNOSTIC TOOLS

We have examined the potential for diagnostic as well as prognostic tools using exosomes. There has been an explosion in this area over the last ten years as "tools" for separating exosomes and examining their contents have advanced. It is my opinion that the ongoing ability to develop these tools is essential but that the process is iterative. Tools allow for the examination of what one is looking for but they also open the process by seeing new issues which need examination. Thus this will perforce become an iterative and ongoing process.

10.3 IMMUNOTHERAPEUTIC HORIZONS

One of the interesting approaches is the use of toll like receptors as found in the innate immune system. If one accepts the Neo Classical model, then it opens the door for examining the attack on exosomes, not just the cancerous or malignant cell. In addition an early attack may prevent metastasis.

10.4 DATA ASSEMBLY

There clearly is a driving need to better understand and structure the Neo-Classical model. If we were to assume that miRNAs are the main drivers then it is essential to determine which of them are the promoters of metastatic behavior. What genes do the suppress, what histones are modified. Is it just one miRNA or is it a pattern. Will one mutation send out an initial miRNA and start the ball rolling and then await one or several more?

To understand this is a massive data collection and management process. Specifically, what are the best miRNA for promoting metastatic growth and why? What genes do the activate or suppress.

For a large scale data bank one would need to match miRNAs in exosomes to specific RNAs in a cancer cell or in a benign cell which can be changed to a cancerous one. For example if the miRNA blocks an apoptosis path, excites a proliferation path, excites a vascularization path, and so forth one can then attempt to map out potential targets for therapeutic development.

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