NOTCH Pathway, miR-146a and Melanoma

Terrence P McGarty
White Paper No 114
June, 2014

Recent studies have recognized the key role a micro RNA, miR-146a, has in the development of melanoma. This paper examines that work and uses it as another window onto the ever increasing incidence of miRNAs in cancer.

Notice

This document represents the personal opinion of the author and is not meant to be in any way the offering of medical advice or otherwise. It represents solely an analysis by the author of certain data which is generally available. The author furthermore makes no representations that the data available in the referenced papers is free from error. The Author also does not represent in any manner or fashion that the documents and information contained herein can be used other than for expressing the opinions of the Author. Any use made and actions resulting directly or otherwise from any of the documents, information, analyses, or data or otherwise is the sole responsibility of the user and The Author expressly takes no liability for any direct or indirect losses, harm, damage or otherwise resulting from the use or reliance upon any of the Author's opinions as herein expressed. There is no representation by The Author, express or otherwise, that the materials contained herein are investment advice, business advice, legal advice, medical advice or in any way should be relied upon by anyone for any purpose. The Author does not provide any financial, investment, medical, legal or similar advice in this document or in its publications on any related Internet sites.

Contents

1 INTRODUCTION

The understanding of cancer progression has been a continuously evolving process moving from internal pathway elements, to micro RNA interactions and to a complete set of epigenetic factors including various methylation effects. We consider here a recent observation of a micro RNA initiated by change in a pathway element, BRAF V600, and its effect on proliferation and survival. This work by Forloni et al introduces putative new elements to block with a putative therapeutic result, one in fact that handles a multiplicity of factors.

In a recent paper by Forloni et al the authors state:

Oncogenic mutations in BRAF and NRAS occur in 70% of melanomas. In this study, we identify a specific microRNA, miR-146a, which is highly upregulated by oncogenic BRAF and NRAS. Expression of miR-146a increases the ability of human melanoma cells to proliferate in culture and form tumors in mice, whereas knockdown of miR-146a has the opposite effects. We show these oncogenic activities are due to miR-146a targeting the NUMB mRNA, a repressor of Notch signaling.

The focus is now clearly on these secondary factors, namely the micro RNAs and even methylation effects that are seen in many cancers. In this case it of the excess production of a specific miRNA that in turn block an mRNA and in turn allows upregulation of other pathways and in turn unregulated cell proliferation.

As Garraway states in NEJM:

Finally, these findings invite speculation that adding γ-secretase inhibitors to inhibitors of RAF and MEK might offer an attractive therapeutic cocktail for assessment in future clinical trials of melanoma treatment. Given the substantial toxicity of γ-secretase inhibitors, additional preclinical studies of such combinations in melanoma cell lines and patient derived xenograft models would be beneficial.

Such studies could clarify the generalizability of Notch dependency in melanoma, the relevance (if any) of the pre miR146a G allele versus the C allele for patient stratification, and the possible usefulness of alternative dosing and scheduling schema to reduce toxicity. Overall, this study provides a reminder that, despite numerous advances, we have only just begun to dissect the rich interplay among noncoding RNAs, the biologic basis of cancer, and potential therapeutic strategies.

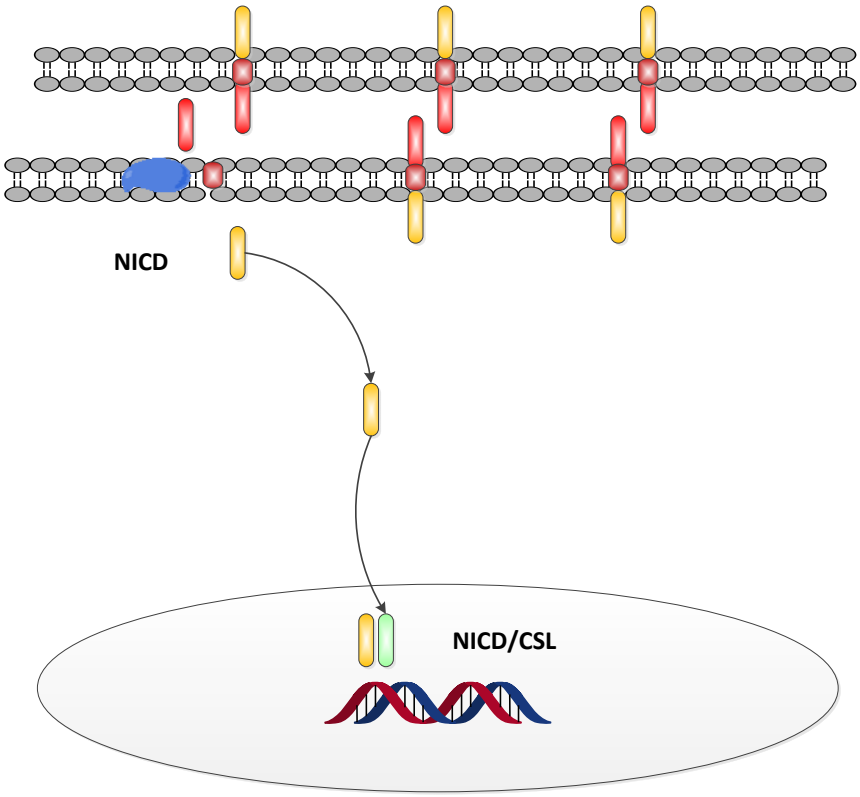
Garraway sees this as a significant breakthrough and believe it is a worthwhile pathway for new therapeutics. In this analysis we briefly examine the interaction of Notch and miR-146a and how it can be understood in the case of melanoma as a major factor of uncontrolled proliferation.

2 NOTCH PATHWAY

Proteolysis is the process of degradation of proteins in the cell and the release of the energy contained therein for other purposes. The Notch pathway process is a key part of the proteolysis effort[1]. The Notch system is a proteolytic driven system used in signal transduction in cells. Uncontrolled Notch pathways production can lead to uncontrolled cellular growth.

2.1 THE PATHWAY

Let us begin with a simplified but reflective description of the Notch pathway. The Notch process starts with the two Notch ligands, which are also called DSL proteins. One is external to the cell membrane and is the other is internal. When they are broken, the intracellular part, called NICD moves to the nucleus and binds with a protein CSL which becomes a putative transcription factor. We demonstrate that below.



Recall, that a transcription factor is a protein or protein complex that can turn on (activators) or turn off (repressors) the transcription of genes[2]. In this case the transcription factor is an activator

[1] See Marks, Chapter 13.

[2] See Broad https://www.broadinstitute.org/education/glossary/transcription-factor and Watson et al 544-555. Also http://www.nature.com/scitable/definition/general-transcription-factor-transcription-factor-167 Also from Vaquerizas:

for MYC[3]. Transcription factors are frequently brought to bear to activate genes that lead to uncontrolled growth.

Goss and Kahn have presented a review of the interaction of Notch and Wnt and especially the function of excess Notch activation as a part of cell proliferation in multiple cancers[4]. As they state Wnt and Notch act in concert in many cancers, prostate being one which we have examined in some detail. In addition excess Notch activation appears to effect a stem cell like behavior in these cells thus resembling the cell types enable for proliferation as well as survival.

2.2 FACTORS INVOLVED

We now want to explore some of the impacts of Notch in stem cell environments and in turn in the maturation of cells. We focus on a recent paper by Katoh and Katoh. As Katoh and Katoh have written:

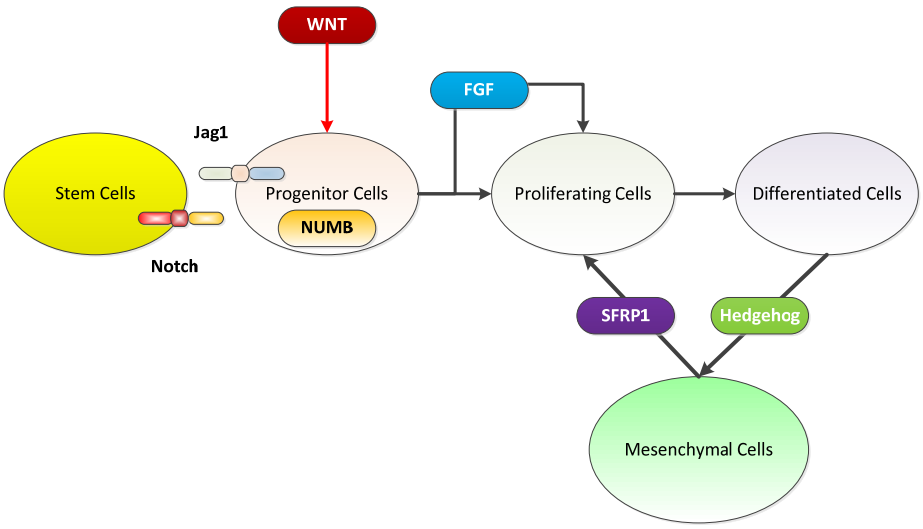
Notch signaling pathway is implicated in the maintenance of self-renewal potential in stem cells, binary cell-fate determination in progenitor cells, and induction of terminal differentiation in proliferating cells. Notch-ligand binding to Notch receptors leads to the cleavage of Notch receptors by metalloprotease and Á-secretase to induce nuclear translocation of Notch intracellular domain (NICD). Nuclear complex, consisting of CSL (RBPSUH), NICD, Mastermind (MAML), p300 and histone acetyltransferase (HAT), then induces transcriptional activation of Notch target genes, such as HES1, HES5, HES7, HEY1, HEY2 and HEYL. HES/HEY family members are bHLH-type transcriptional repressors for tissue-specific transcription factors. Therefore, Notch signaling activation in stem cells leads to the maintenance of self-renewal potential.

Now Katoh and Katoh provide an activation path progression as show below (as modified):

"Transcription factors are key cellular components that control gene expression: their activities determine how cells function and respond to the environment. Currently, there is great interest in research into human transcriptional regulation. However, surprisingly little is known about these regulators themselves. For example, how many transcription factors does the human genome contain? How are they expressed in different tissues? Are they evolutionarily conserved? Here, we present an analysis of 1,391 manually curated sequence-specific DNA-binding transcription factors, their functions, genomic organization and evolutionary conservation. Much remains to be explored, but this study provides a solid foundation for future investigations to elucidate regulatory mechanisms underlying diverse mammalian biological processes."

[3] From NCBI we have: T*he protein encoded by this gene, cMYC, is a multifunctional, nuclear phosphoprotein that plays a role in cell cycle progression, apoptosis and cellular transformation.* ***It functions as a transcription factor that regulates transcription of specific target genes.*** *Mutations, overexpression, rearrangement and translocation of this gene have been associated with a variety of hematopoietic tumors, leukemias and lymphomas, including Burkitt lymphoma. There is evidence to show that alternative translation initiations from an upstream, in-frame non-AUG (CUG) and a downstream AUG start site result in the production of two isoforms with distinct N-termini. The synthesis of non-AUG initiated protein is suppressed in Burkitt's lymphomas, suggesting its importance in the normal function of this gene.* See http://www.ncbi.nlm.nih.gov/gene/4609

[4] See Goss and Kahn, pp60-61.



From Katoh and Katoh: WNT and Notch signaling networks for the homeostasis of stem and progenitor cells. Canonical WNT activates the β-catenin - TCF signaling cascade to induce NUMB and JAG1 expression in progenitor cells. NUMB inhibits Notch signaling in progenitor cells to induce differentiation, while JAG1 activates Notch signaling in stem cells to maintain self-renewal potential.

The above demonstrates the progress from an overactive Notch cell which thus acts as a stem cell to more mature cell lines. The above also demonstrates the location of proliferating cells in this schema, just after the stem cell line progenitor.

Thus the activation of Notch leads to an extreme survival capability in cells so activated. They continue with the following regarding NUMB:

NUMB and NUMB-like (NUMBL), consisting of phosphotyrosine-binding (PTB) domain and SH3-binding proline-rich region, are docking proteins functioning as Notch signaling inhibitors. Here, we searched for the TCF/LEF-binding site within NUMB and NUMBL promoters. Because two TCF/LEF-binding sites were identified within human NUMB promoter, comparative integromics analyses on NUMB orthologs were further performed.

Thus one way to over-activate Notch is to suppress NUMB. NUMB is described by NCBI as follows[5]:

The protein encoded by this gene plays a role in the determination of cell fates during development. The encoded protein, whose degradation is induced in a proteasome-dependent manner by MDM2, is a membrane-bound protein that has been shown to associate with EPS15, LNX1, and NOTCH1.

In a similar manner NOTCH1 is described as follows[6]:

[5] http://www.ncbi.nlm.nih.gov/gene/8650

[6] http://www.ncbi.nlm.nih.gov/gene/4851

This gene encodes a member of the Notch family. Members of this Type 1 transmembrane protein family share structural characteristics including an extracellular domain consisting of multiple epidermal growth factor-like (EGF) repeats, and an intracellular domain consisting of multiple, different domain types. Notch family members play a role in a variety of developmental processes by controlling cell fate decisions.

The Notch signaling network is an evolutionarily conserved intercellular signaling pathway which regulates interactions between physically adjacent cells. ...Homologues of the notch-ligands have also been identified in human, but precise interactions between these ligands and the human notch homologues remain to be determined. This protein is cleaved in the trans-Golgi network, and presented on the cell surface as a heterodimer. This protein functions as a receptor for membrane bound ligands, and may play multiple roles during development.

These are two powerful and interacting genes. NUMB suppresses Notch1 and Notch1 when activated makes for cell proliferation and survival.

3 MIR-146A

There are now well over hundreds of micro RNAs, which a small non-coding RNAs which result in the control of various pathways in cellular signalling. Micro RNAs are often encoded in introns in mRNAs and some in in non-coding RNAs. They generally control mRNA in terms of its stability, degradation and/or translation. The micro RNAs can stop genes from being expressed as proteins, even though the gene is present and provides a normal mRNA. They are small, generally 22 base pairs in length.

3.1 SPECIFICS OF MIRNA

As NCBI states[7]:

microRNAs (miRNAs) are short (20-24 nt) non-coding RNAs that are involved in post-transcriptional regulation of gene expression in multicellular organisms by affecting both the stability and translation of mRNAs. miRNAs are transcribed by RNA polymerase II as part of capped and polyadenylated primary transcripts (pri-miRNAs) that can be either protein-coding or non-coding.

The primary transcript is cleaved by the Drosha ribonuclease III enzyme to produce an approximately 70-nt stem-loop precursor miRNA (pre-miRNA), which is further cleaved by the cytoplasmic Dicer ribonuclease to generate the mature miRNA and antisense miRNA star (miRNA) products. The mature miRNA is incorporated into a RNA-induced silencing complex (RISC), which recognizes target mRNAs through imperfect base pairing with the miRNA and most commonly results in translational inhibition or destabilization of the target mRNA.*

As Rusca and Monticelli state:

Initial evidences on the possible involvement of miR- 146a in cancer came from a study showing thatmiR-146a was upregulated in papillary thyroid carcinoma (PTC) samples compared with unaffected thyroid tissue. Interestingly, a set of five miRNAs, including miR-221, miR-222, and miR- 146, was sufficient to distinguish unequivocally between PTC and normal thyroid. Similarly to the observations performed in immunologic settings, overexpression of miR- 146a/b in the highly metastatic human breast cancer cell line MDA-MB-231 significantly downregulated expression of IRAK1 and TRAF6, negatively regulating NF-κB activity. Functionally, this resulted in markedly impaired invasion and migration capacity relative to control cells.

These findings implicated miR-146 not only as a negative regulator of constitutive NF-κB activity in breast cancer cells, but also suggested that modulating miR-146 levels might have therapeutic potential to suppress breast cancer metastases. Along the same line, miR-146a was among the miRNAs found upregulated in cervical cancer tissues compared to normal cervix.

[7] http://www.ncbi.nlm.nih.gov/gene/406938

When introduced into cell lines, miR- 146a promoted cell proliferation. Although the molecular mechanism underlying such increased proliferation remains to be investigated, these observations potentially implicate miR-146a in cervical carcinogenesis. In another type of cancer, the hormone-refractory prostate carcinoma (HRPC), miR-146a levels were diminished compared to androgen-sensitive noncancerous epithelium. In this context, miR- 146a acted as a tumor suppressor, reducing levels of its target ROCK1, one of the key kinases involved in HRPC transformation.

Accordingly, forced miR-146a expression reduced ROCK1 protein levels, cell proliferation, invasion, and metastasis to human bone marrow endothelial cell monolayers. Similarly, miR-146a was lower in pancreatic cancer cells compared with normal human pancreatic cells…

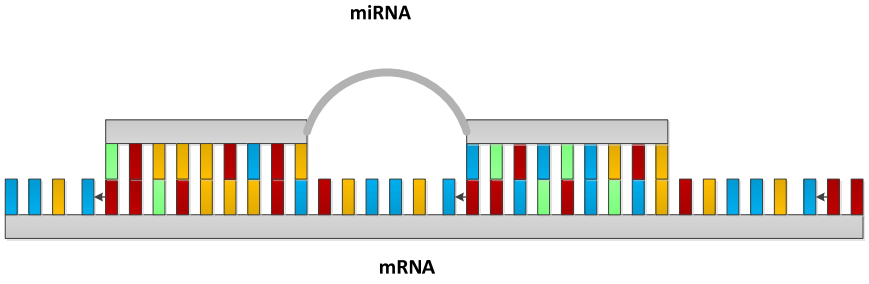
There is now increasing evidence to suggest that miR-146a is involved in the regulation of the adaptive as well as innate immune response, and that miR-146a can be an important player in regulating tumor progression.

However, more work remains to be done to fully understand its role and mechanism of action in normal and pathologic conditions, so that expression of this miRNA can potentially be exploited as a new point of entry for therapy. With the identification of a vast number of miRNAs each carrying a long list of putative targets, the challenge is now to understand the details of their biological functions.

Thus miR-146a has significant roles to play in controlling cell behavior.

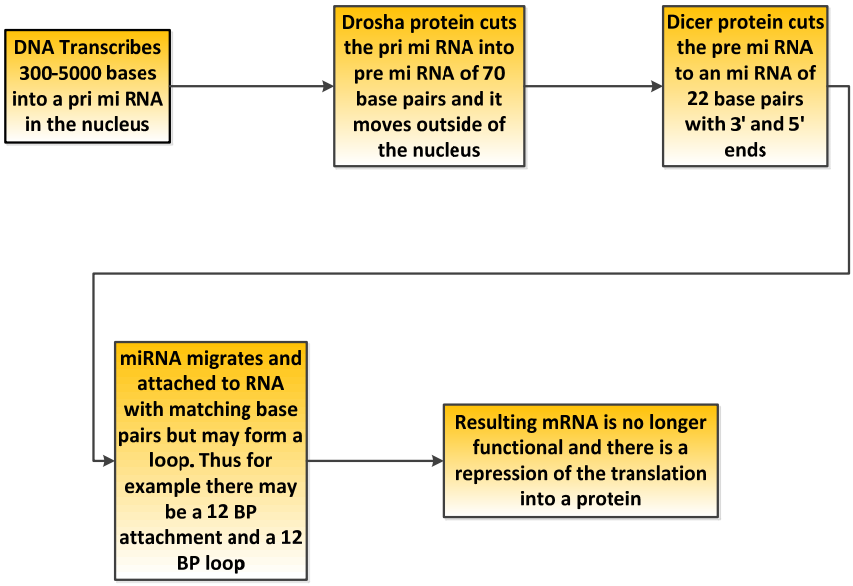
3.2 MIRNA DYNAMICS

For example, miRNAs can inhibit the translation of mRNA into a protein. We show this below. The small segment attaches to the mRNA and blocks translation. This graphic is descriptive and does not contain full details[8].

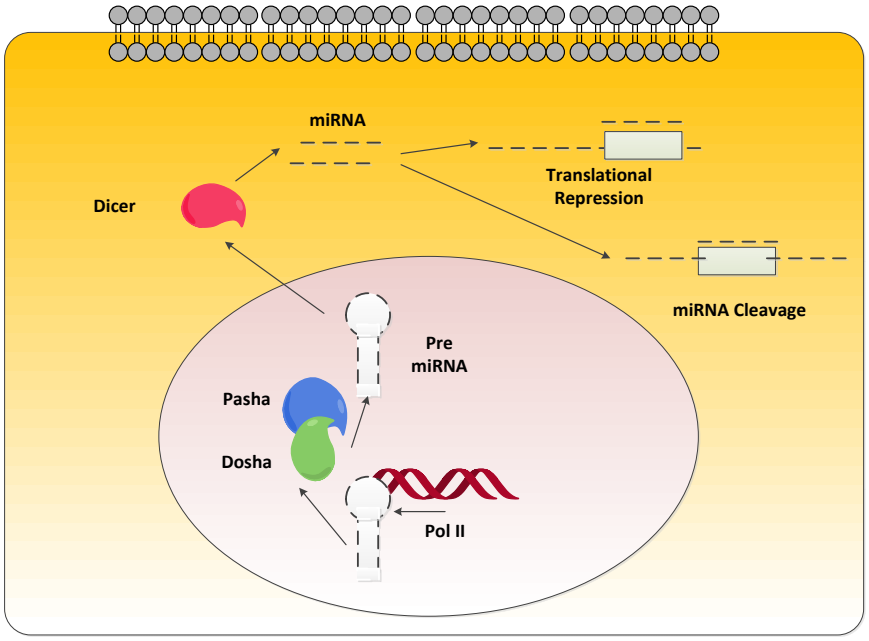


[8] See Marks p 318. Note the colors are also descriptive and do not reflect any specific RNA base pair pairing. Just as with DNA we would expect similar bonding of CG and A and U.

In the case being discussed, miR-146a binds to NUMB and suppresses it. That in turn allows for an overexpression of Notch which in turn can lead to an unstable system with feedback. We shall detail that a bit later.

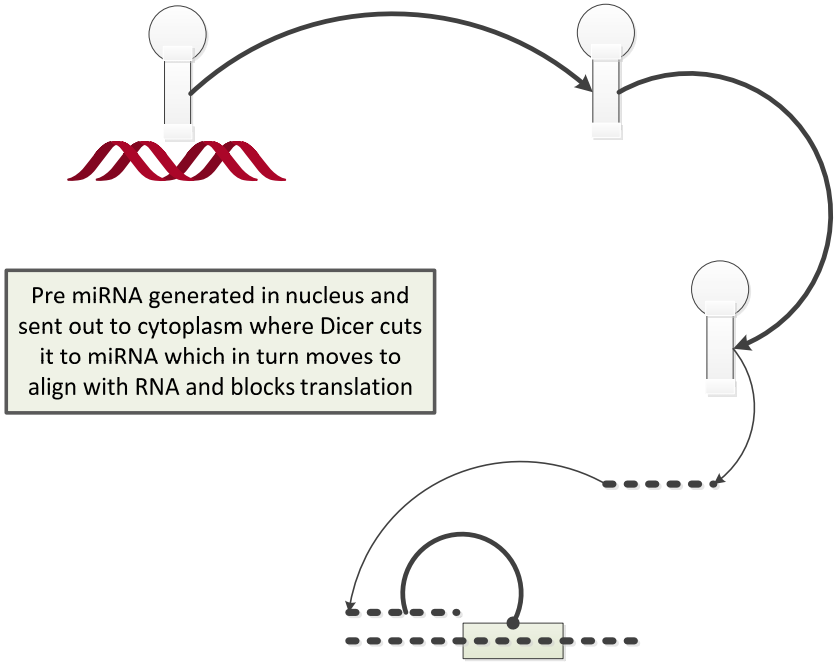


We depict that process in some detail below. For the most part all miRNAs appear to function in the same manner. There are well over a thousand identified at this point and more than likely many more to be found. The functions of most are not fully known.

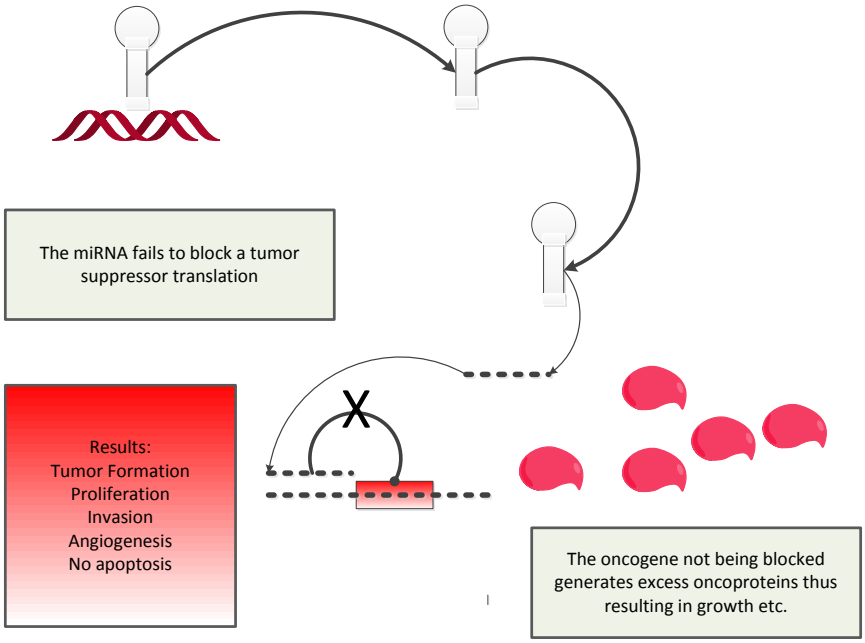


3.3 MIRNA INTERACTIONS

Before continuing it is worth a quick review of normal and abnormal behavior of miRNA. The normal process is shown below. This shows a classic blocking of translation. The miRNA binds to the mRNA and inhibits translation. The question is what makes the miRNA to do this? Namely what forces the generation of the miRNA? Is it a random effect or is it part of a planned process. We have shown that homeostasis is well defined in terms of a balanced expression of RNA. Yet when we have an aberrant genetic element the miRNA can express itself in deleterious ways.

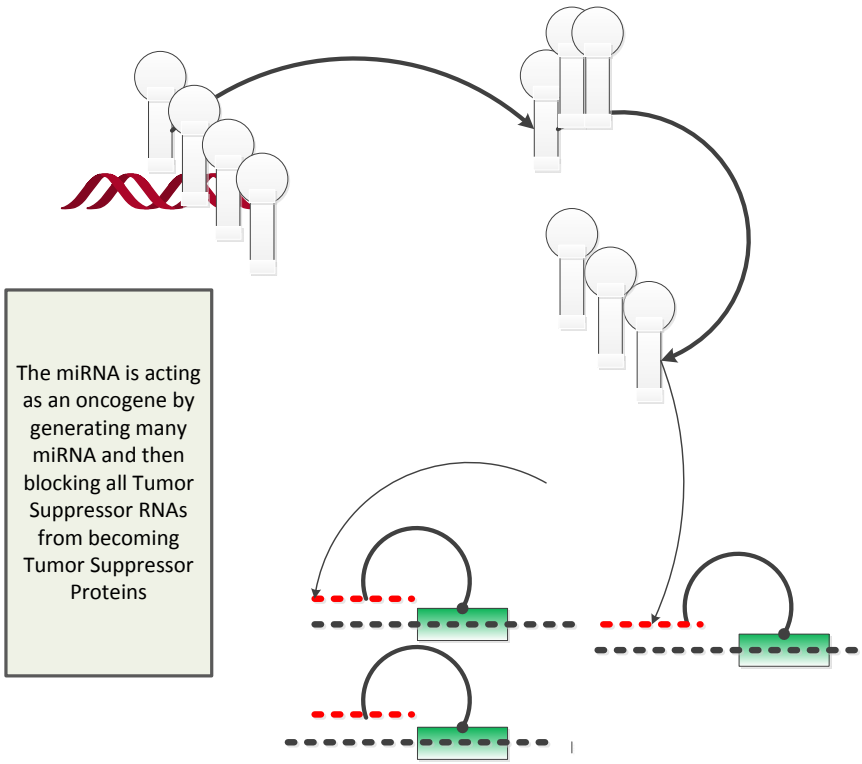


Now we can examine a miRNA in the context of a cancerous environment shown below. The diagram below shows miRNA blocking a tumor suppressor gene. In a sense the example of the miR-146a is an example of this type of miRNA operation. It blocks a protein which in turn blocks a protein which leads to unbridled growth and survival, Notch.



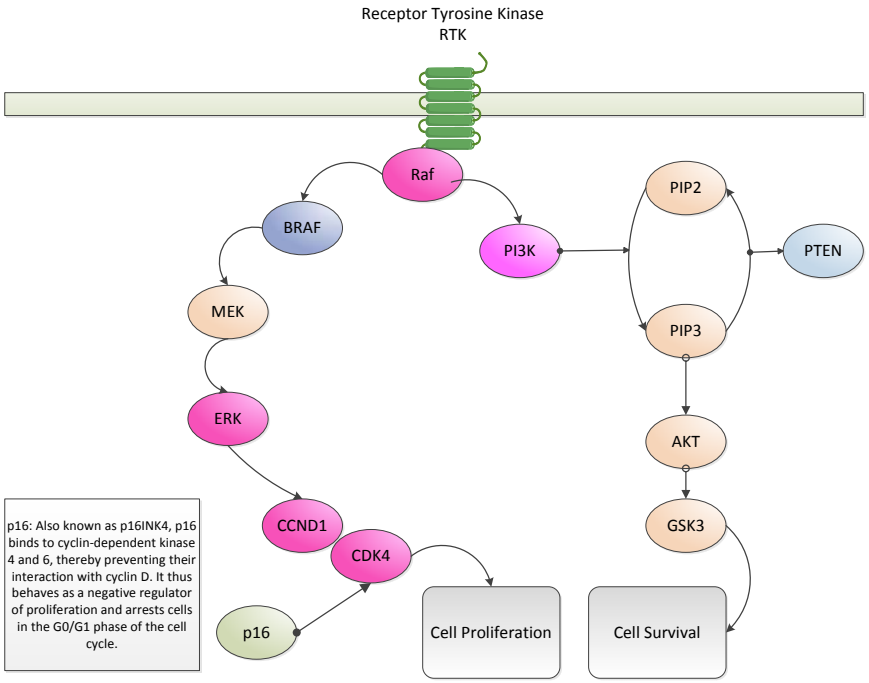
Finally we show the example of miRNA in some explosive expansion of itself thus blocking many tumor suppressor genes. This is a deadly mode for miRNAs and can be found in many cancers[9].

[9] It is worth examining the McGarty DRAFTs on Prostate Cancer and Melanoma to see this in some detail.


The miRNA is acting as an oncogene by generating many miRNA and then blocking all Tumor Suppressor RNAs from becoming Tumor Suppressor Proteins

4 MELANOMA TARGETING

The classic pathway dynamics we understand regarding proliferation and survival is shown below. This is the BRAF and PI3K dynamics. We demonstrate this below. This is a well-known and well understood pathway and is at the core of the BRAF V600 therapeutic approach.



Now proliferation and survival require gene activation and maintenance.

In this report, we demonstrate a critical role for miR-146a in the initiation and progression of BRAF/ NRAS-positive melanomas, ... In addition, our results reveal a pharmacologically tractable pathway for the treatment of melanoma. We identified miR-146a as the microRNA whose expression was most upregulated by activated BRAF.

Upregulation of miR-146a by activated BRAF, as well as activated NRAS, occurs through the MAPK signaling pathway. Accordingly, we find that BRAF and NRAS mutant melanoma cell lines and short-term melanoma cultures show higher levels of miR-146a compared to those that are wild type for these genes.

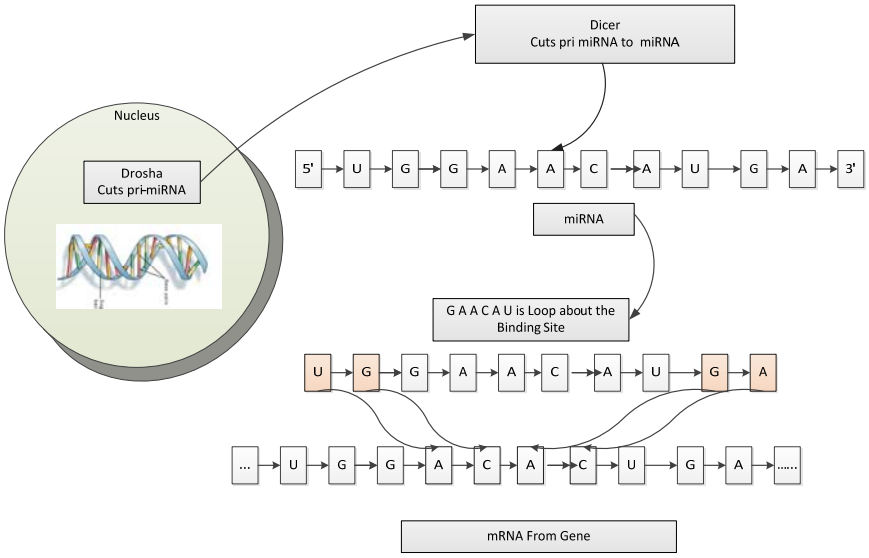
A major function of the MAPK pathway is to activate transcription by regulating the stability and expression of multiple transcription factors primarily through direct phosphorylation.

We show that the MAPK pathway regulates the phosphorylation of the transcription factor MYC, which in turn binds to the promoter of miR-146a and stimulates its transcription. Notably, MYC has been found to stimulate transcription of several other miRNAs. For example, MYC has been shown to directly activate transcription of the oncogenic miR-17-92 cluster and thereby promote

cell proliferation, survival, angiogenesis, and metabolic reprogramming in a number of tumor cell lines.

miRNAs and components of miRNA biogenesis pathways such as Dicer have been implicated in several aspects of melanocyte biology as well as in melanoma initiation and progression.

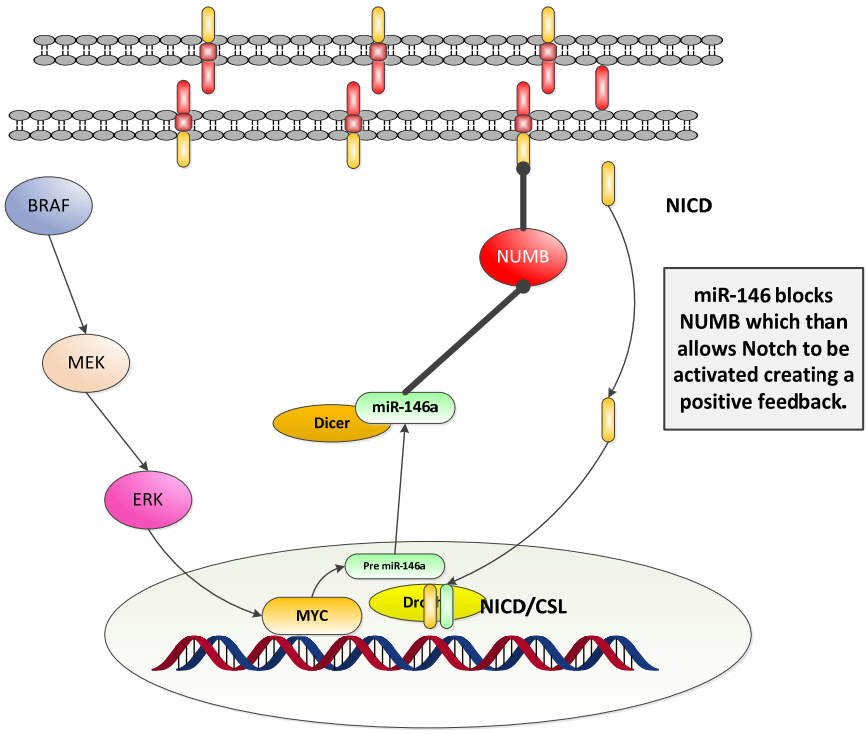
We depict some of that process below:



Previous studies have shown that miR-146a can function either as an oncogene or as a tumor suppressor depending upon the cell type. For example, miR-146a has been shown to function as an oncogene in a variety of human cancers including papillary thyroid carcinoma (PTC), triple negative sporadic breast cancers and anaplastic thyroid carcinoma. miRNAs function primarily by targeting mRNAs and either promoting their degradation or blocking their translation.

Our analysis identified 20 potential targets of miR-146a, including NUMB, which is a well-characterized Notch signaling inhibitor. It is thought that NUMB negatively regulates NOTCH, potentially through a direct protein–protein interaction that requires the phosphotyrosine-binding (PTB) domain of NUMB and either the RAM23 region or the very C-terminal end of NOTCH.

We demonstrate some of these dynamics in the Figure below (adapted from Galloway with modifications).



In simple terms:

1. BRAF activates MEK
2. and then ERK
3. which activates MYC
4. which activates pre miR-146a and
5. then via Drosha and Dicer makes miR-146a
6. which reactivates Notch by suppressing NUMB expression (we have left that out for simplicity)
7. which then goes down to the transcription on the DNA resulting in proliferation and stem like behavior.

This is an interesting and compelling mechanism for the explanation of the aggressive melanoma expansion.

5 OBSERVATIONS

This is an interesting step in the understanding of melanoma genomics. The role of micro RNAs is becoming clearer as time goes by and added to that is the effect of such epigenetic factors as methylation and we now see a much more complex field of play than a decade ago. The benefit is the recognition of more targets of opportunity that can be had for potential therapeutics. On the other hand the main concern is that the more that is learned one may ask what else is there yet to grasp.

Thus what observations can we make here? Let us examine a few:

1. Stem Cell Hypothesis. Here we have the elements of how a stem cell functions with the activated Notch and blocked NUMB. Does this imply that we have the re-emergence of stem cell like malignant cells activated in a manner such as this. Namely the miRNAs allow for the reprogramming of some modified form of totipotency.

2. Targeted Therapeutics: Galloway makes this observation We know that BRAF inhibitors get us one step there but then we need MEK inhibitors. Then what? Does an inhibitor for miR-146a take all the steps necessary or does the cell go and find another back door way to function?

3. The Dynamics of the Processes are Not Well Understood. One of the problems in understanding the impact of miRNAs and other pathway elements is that there is a concern as to the number or concentration of products. If miR-146a is to block NUMB then it should block all NUMB and in turn activate all Notch. Yet it is a molecule by molecule process which seems to be poorly understood. The paper by Choir et al and Nazarov et al present some ideas on how to deal with such issues. However they are but first steps. This is a critical factor to understand since the therapeutics depends on blocking the necessary number of miR-146a molecules. To data there seems to be limited data to assess this issue.

4. Initiation and Support: We know that V600 mutation of BRAF is drivers for metastatic melanoma. However it is not clear what is the driver ultimately for miR-146a, although it appears as we have suggested as a sequella from the other mutations. Additional insight into the proliferation is requires.

6 REFERENCES

1. Appasani, K., MicroRNAs, Cambridge (New York) 2008.
2. Broad Institute, https://www.broadinstitute.org/education/glossary
3. Choi, M., et al, A dynamic expression survey identifies transcription factors relevant in mouse digestive tract development, Development 133, 4119-4129 (2006) doi:10.1242/dev.02537
4. Forloni, M., et al, miR-146a promotes the initiation and progression of melanoma by activating Notch signaling, eLife 2014;3: e01460. DOI: 10.7554/eLife.01460
5. Garraway, L., A Notch for Noncoding RNA in Melanoma, NEJM, 370;20, May 15, 2014.
6. Goss, K., M. Kahn, Targeting the Wnt Pathway in Cancer, Springer (New York) 2011.
7. Katoh, M., M., Katoh, NUMB is a break of WNT - Notch signaling cycle, INTERNATIONAL JOURNAL OF MOLECULAR MEDICINE 18: 517-521, 2006
8. Lawrie, C., MicroRNAs in Medicine, Wiley (Hoboken) 2014.
9. Marks, F., et al, Cellular Signal Processing, Garland 2008.
10. McGarty, T., Melanoma Genomics, DRAFT 2013.
11. McGarty, T., Prostate Cancer Genomics, DRAFT, 2013.
12. Nazarov, P., et al, Interplay of microRNAs, transcription factors and target genes: linking dynamic expression changes to function, Nucleic Acids Research, 2013, Vol. 41, No. 5 2817–2831.
13. Rusca, S., C. Monticelli, MiR-146a in Immunity and Disease, Molecular Biology International, Volume 2011, Article ID 437301, 7 pages
14. Sangunitti, et al, Probabilistic inference of transcription factor concentrations and gene-specific regulatory activities, Bioinformatics, Vol. 22 no. 22 2006, pages 2775–2781
15. Vaquerizas, J., et al, A census of human transcription factors: function, expression and evolution, Nature Reviews Genetics 10, 252-263 (April 2009) | doi:10.1038/nrg2538.
16. Watson, J., et al, Molecular Biology of the Gene, 5th Ed, Benjamin (San Francisco) 2004.