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Review

The miRNA-9 Isoform Story in Cancer: An OncomiR or Tumor Suppressor?

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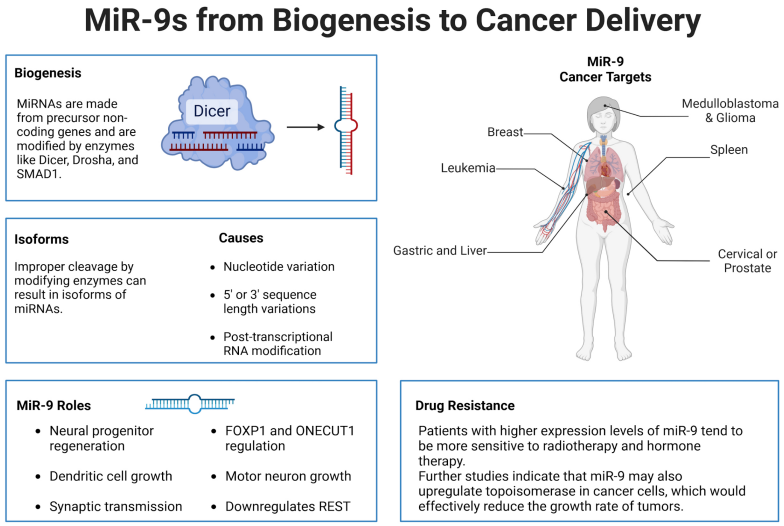
Abstract

MicroRNAs (miRNAs) are small, non-coding RNA molecules that regulate gene expression post-transcriptionally. They function by binding to newly transcribed mRNAs of protein-coding genes, suppressing gene expression at the post-transcriptional level. Thus, miRNAs play a crucial role in controlling a plethora of cellular processes, making miRNAs a unique class of regulatory RNA with defined developmental roles. Thus, these small yet powerful miRNAs have garnered the attention of many researchers due to the known biological regulatory role during chronic disease onset, including cancer. Over the past decade, novel small RNA therapies have been developed and implemented successfully in the clinic to combat several chronic disorders. With respect to cancer, a number of miRNAs were demonstrated to be correlated with cancer progression and disease onset. These oncomiRs or tumor suppressors control well conserved protein signaling cascades that become dysregulated during the tumorigenic process. In particular miR-9 has garnered much attention over the past decade due to the abundance of this miRNA in certain tissues such as the brain and the epithelium. In this review, we discuss the importance of miRNAs in cancer, the biology of miR-9 in a variety of cancers, explain how the term 'oncomiR' or 'tumor suppressor miR' depends upon the cellular context of gene expression during oncogenesis, and how the abundance of certain miR-9 isoforms in the cell during the initiating tumorigenic event could influence the molecular heterogeneity of the tumor. Additionally, next-generation miRNA therapeutics offer promising strategies for cancer treatment by the precise targeting of disease-related pathways with minimal toxicity and off-target effects. These strategies can also serve as solutions in situations where chemo- and radio-resistance persists.

Graphical Abstract



Graphical Abstract. An Overview of miR-9 Biogenesis and Function During Tumorigenesis.

miRNAs are powerful regulators of gene expression in mammalian systems. miRNAs are processed in the cell by Drosha and Dicer that results in an 18-22nt duplex RNA. This duplex gets unwound by AGO proteins where one strand is selected to mediate cellular function by binding to cognate target mRNAs through Watson-Crick base pairing. Many times Dicer cleavage may be incomplete, or the sequence of the miRNA originating from a separate genomic loci may contain some repetitive sequence near the 5' or 3' ends of the eventual miRNA duplex. These miRNA isoforms can influence strand selection of the duplex, ultimately affecting which mRNAs are targeted for degradation. As an example, miR-9 has various known isoforms, with each mature strand controlling different components of neural progenitor regeneration, synaptic transmission, and even drug resistance in cancer.

1. Introduction

MicroRNAs (miRNAs) regulate gene expression patterns during normal developmental processes, as best exemplified during normal *C. elegans* larval development [1-4]. From these early studies researchers elucidated that the dysregulation of even a single miRNA can have drastic effects on the abundance of hundreds of cognate target mRNA species [5-8]. In diseases such as cancer, miRNAs have been classified simply as oncomiRs or tumor suppressors based on the regulatory actions imposed on then known protein-coding tumor suppressor genes and oncogenes, respectively. However, this oversimplification of miRNA function has limited our understanding of how these complex regulatory agents control the signaling pathways that influence the tumorigenic process. Furthermore, despite the efforts of research scientists over the past four decades, neoplasms still remain a top cause of mortality worldwide [9-11]. Therefore understanding new genetic pathways, such as miRNA and other non-coding RNAs [12], may yield novel RNA-based therapeutics with the potential to cure individuals that have cancer. Returning to miRNA function, firstly, over half of the known miRNA genes are located within genomic regions associated with cancer, or within genomic fragile sites prone to chromosomal breakpoints [13-15]. Fragile sites, chromosomal breakpoints, and translocations present within hematological tumors were where some of the the first cancer-associated miRNAs were functionally identified. From there, early cloning and deep sequence studies identified thousands of abundant, mature, 18-22 nucleotide miRNA sequences with unknown function [16-19]. The abnormal expression patterns of these miRNA species across many cancer types indicated that miRNAs contributed to, or at least correlated with, cancer progression. After decades of research, the methodology to identify these miRNA have developed significantly, from early microarray expression technology, to complex strategies that capture and deep sequence both miRNAs and miRNA sequence variants. This process allowed for the generation of tissue-specific small RNA libraries from limited biospecimen sources, such as tumor biopsies, serum, cerebrospinal fluid, and dermal tissue punch biopsies [20]. These strategies have allowed researchers to further elucidate how non-coding RNAs profoundly impact disease onset, development, progression, and drug sensitivity.

From the early research studies in the field of liquid and blood-related tumors, a small handful of miRNAs were identified to have direct regulatory action during leukemogenesis. Once researchers studying cancers of the epithelium from organ sites, such as the prostate, breast, gastric, lung, and thyroid, appreciated the involvement of miRNAs in cancer, hundreds of conserved miRNA-protein coding gene networks have been identified to directly contribute to the development of tissue-specific carcinomas [21-26]. Again, the first evidence that miRNAs were dysregulated in cancer stemmed from the work of Croce and Calin *et. al.*, who identified miRNA dysregulation was present in chronic lymphocytic leukemia (CLL) [27-31]. Their pioneering research in the early 2000s revealed that deletions on chromosome 13q14.3 led to the loss of miR-15a and miR-16-1. These miRNAs directly bind and target anti-apoptotic genes such as BCL2. Therefore, the loss of miR-15a and miR-16-1 results in enhanced BCL2 expression, thereby promoting tumor cell survival [32-34]. This discovery not only highlighted the tumor

suppressive role of certain miRNAs, but also paved the way for researchers to continue elucidating the involvement of miRNAs within hematological malignancies as well as in epithelial carcinomas.

MiRNA research has yielded promising clinical diagnostics due to the production of biomolecules that can serve as both therapeutic agents as well as disease biomarkers [35-38]. The astounding breakthroughs in RNA chemistry over the past decade have allowed researchers to develop RNA based compounds that have superior clinical benefit when compared to standard gene therapy approaches. For the miRNA field, scientists have found that tumor tissues often display noticeably reduced expression of certain miRNAs, therefore, miRNA mimics are being developed to restore miRNA levels in cancer patients. Additionally, when miRNAs are found to be in over abundance and contribute to tumorigenesis, miRNA sponges are being developed to disrupt the activity of these miRNAs that support tumorigenesis. The quality of these synthetic small RNA molecules depends on our understanding of the mechanisms that cause cessation or overabundance of miRNA expression, which includes genetic alteration, epigenetic silencing, and miRNA biogenesis pathway defects.

Yet how do miRNAs truly function? As will be explained in more detail below, miRNAs are small endogenous non-coding RNAs, usually 18-22 nucleotides long, that negatively regulate protein-coding gene expression post-transcriptionally (see Figure 1). Importantly, miRNAs are necessary for the regulation of gene expression and fine tuning of almost all biological processes [39-44]. The genes that encode a mature miRNA sequence begin with a transcribed region of the genome that yields a primary miRNA (pri-miRNAs). This polycistronic sequence is then sequentially processed by a series of RNAseIII catalytic enzymes, such as DROSHA, into precursor miRNAs (pre-miRNAs) of around 70-80 nucleotides. Sequentially, the RNAseIII enzyme DICER, converts these pre-miRNAs to a mature miRNA sequence of around 19-23 nucleotides [40,45-47]. The miRNA duplex undergoes an event called strand selection, whereby one strand of the duplex is bound to an RNA-induced silencing complex, which is then guided to the 3' of the untranslated region (UTR) of a cognate target mRNA (see Figure 1). This interaction results in the post transcriptional repression of a cognate mRNA target, or the direct cleavage and degradation of the mRNA target.

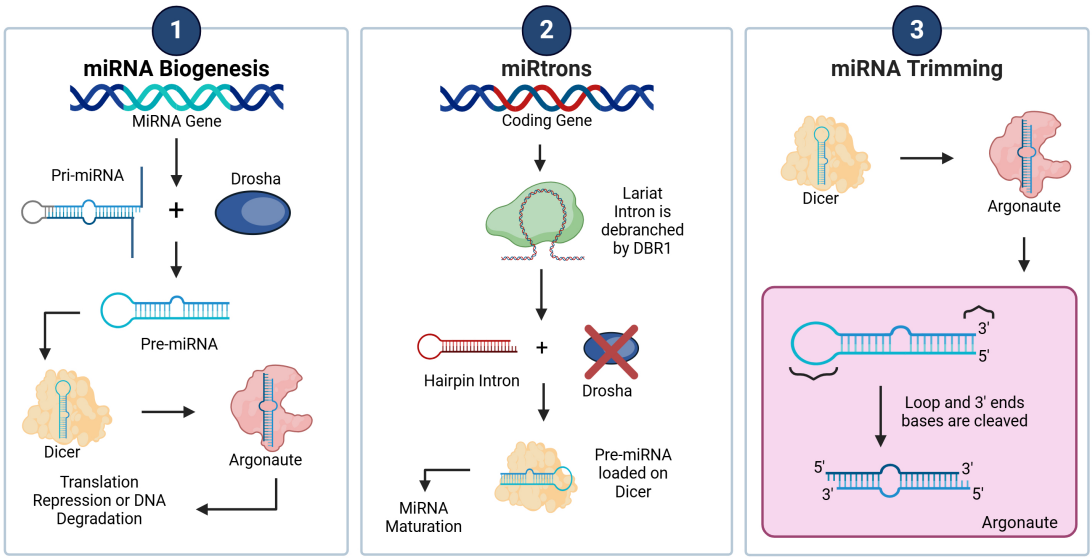


Figure 1. The Various Mechanisms that Modulate the Generation of the Final miRNA Strand.
This figure depicts the numerous ways in which miRNAs can be generated. Panel 1, shows the canonical miRNA biogenesis pathway. Here pri-miRNAs transcribed from the genome are processed by DROSHA into an 85nt stem loop, which is recognized by DICER generating a 22 nt duplex [48-53]. Strand selection occurs after binding to AGO, allowing for target mRNA degradation. In Panel 2, miRNAs are generated from introns of protein coding genes. During splicing and lariat formation, a pre-miRNA is formed that bypasses Drosha [54-56]. When this hairpin is loaded into Dicer, the released hairpin becomes the mature miRNA bypassing strand selection and gets loaded into AGO. In Panel 3, during miRNA biogenesis, both DROSHA and DICER can cleave the pre-miRNA in an offset manner by up to 2-3 nucleotides [57-62]. These trimming errors create miRNA isoforms that affect miRNA-mRNA stability, but could also affect strand selection, as trimming occurs at the 5' and 3' ends of the miRNA.

One should appreciate that miRNA interactions are dynamic and are dependent on a variety of factors such as subcellular localization, strength of the interaction between the miRNA and the cognate mRNA target, as well as the abundance of the miRNA and target mRNAs [63-67]. Additionally, miRNA expression becomes dysregulated in human cancer through faulty miRNA transcription, aberrant DNA methylation, miRNA gene amplification or deletion, as well as defects in miRNA biogenesis [68-71]. The fact that miRNA activity depends upon both a miRNA sequence and protein coding gene abundance, explains why it is difficult for many researchers to pin down the exact causality for a miRNA-related cancerous origin. This hypothesis is of course being compared to a more simplistic understanding of tumorigenesis such as a mutation in one protein coding gene such as *TP53,* or *MYC* that results in a pro-tumorigenic event. In support of this notion,

dysregulation of miRNA abundance and activity has been shown repeatedly to be a key player during cancer oncogenesis by controlling cellular differentiation, sustaining proliferation, evading growth suppressors, and resisting apoptosis [72-79]. An example of this miRNA dysregulation is exemplified during glioma malignancy, where dysregulation and mutation of miRNAs may be related to glioma formation, and furthermore the use of miRNAs are being developed into biomarkers for glioma progression [80-89]. Because of research studies such as this, miRNAs are seen as a potential therapeutic for glioblastoma due to their regulation of many known cancer-associated mRNAs. As mentioned earlier, once identified, the altered miRNAs within a tumor can be therapeutically manipulated resulting in a correction of miRNA activity and subsequently, a restoration of the normal cellular signalling processes [90-93]. The hope for a targeted miRNA therapy has been met with some challenges due to organ specific delivery, yet the discovery of miRNA containing exosomes have helped researchers elucidate new strategies for miRNA delivery in the clinic.

2. Mirna Biogenesis

The biogenesis of miRNAs involves a series of intricate enzymatic RNA processing steps. In most organisms, this is a conserved regulatory process, yet there are some nuanced steps that occur in mammalian systems. As explained earlier, miRNAs are encoded within the genome and are found at multiple genomic loci. These genetic loci are transcribed by RNA polymerase II, as most other protein coding genes, and the resulting transcript is 5’ methyl-guanine capped and 3’ poly-adenylated [47,94]. However, these pri-miRNA transcripts form polycistronic hairpin structures that attract type 2 RNase III enzymes that recognize these double stranded RNA sequence hairpins (see Figure 1). As an example, the DROSHA-DGCR8 complex recognizes the polycistronic hairpins within the pri-miRNA and releases these newly formed pre-miRNAs from the nucleus to the cytoplasm [40,45-47]. Cytoplasmic import of nuclear pre-miRNAs are facilitated by a nuclear pore protein termed exportin-5, which also functions as a protector from nucleolytic attack by other RNase enzymes tasked with preventing the accumulation of nuclear RNAs.

While this is a slightly ambiguous process, these pre-miRNAs are recruited mostly to the cytoplasmic regions near the rough endoplasmic reticulum where protein producing polysomes are found. At this stage the pre-miRNA is recognized and further cleaved by another RNase III endonuclease called DICER [48,49]. This occurs because the DICER endonuclease is able to recognize the 2-nucleotide 3’ overhang of the stem loop, as well as the unpaired terminal stem loop causing cleavage and release of a 19-23-nucleotide mature double strand miRNA duplex (see Figure 1). Specifically researchers identified that the PAZ (PIWI-AGO-ZWILLE) domain of DICER functions as a “molecular ruler”, aiding in miRNA-duplex generation with inset 5’-phosphates and 2-nucleotide 3’ overhangs [50-53,95-97]. This also indicates that DICER-mediated miRNA trimming is the key to ensuring precise mature miRNA length and proper functionality.

Occasionally, miRNA precursors are transcribed with additional nucleotides on each end, resulting in extended transcripts. Usually DROSHA can trim the precursor miRNA molecule to remove these additional nucleotides since the embedded miRNA duplex in the pre-miRNA is guiding the RNaseIII-mediated slicing process [50-52]. However, there are errors during this process and researchers have reported miRNA sequences outside the normal defined lengths, with unknown regulatory effects. Now, if DROHSA and DICER fail to properly trim these miRNA precursors during the biogenesis process, and before duplex strand-selection, a larger miRNA with varied sequences would be produced. This longer than normal miRNA could have altered interactions with target cognate mRNAs, or even fail to be incorporated into the RNA-induced silencing complex altogether. In fact, it has been observed that oversized miRNA tends to be unstable, have altered subcellular localization, and differential interactions with regulatory proteins, which could greatly disrupt normal cellular function [98-100]. The link between improperly trimmed miRNAs and disease such as cancer, have yet to be fully elucidated, and is an exciting area of miRNA research.

Furthermore, dysregulation of key enzymes involved in miRNA processing, such as DROSHA and DICER, have been studied, and are known to disrupt the precise processing of miRNA precursors [101-113]. Additionally, genetic mutations within miRNA sequences or regulatory regions could alter the secondary structures of these enzymes, thereby inhibiting the proper formation of duplex-miRNA strands. Environmental factors, such as cellular stress or exposure to toxins may also affect this miRNA biogenesis process [114,115]. Any disruption in the mature-miRNA duplex could impair these miRNAs from properly loading into the RNA-induced silencing complex (RISC) and subsequently properly targeting the appropriate cognate mRNAs. This disruption in miRNA biogenesis could therefore result in cancer development.

3. Variations in Mirna Biogenesis

Returning to miRNA biogenesis, one very important step is strand selection of the miRNA duplex. As mentioned earlier, a typical miRNA-duplex contains an inset 5’-phosphate and a 2-nucleotide 3’ overhang (see Figure 2). This overhang is important as the strand with more instability on one of the duplexed ends (i.e., less base pairing) gets preferentially loaded into the RISC complex, which contains an Argonaute protein (AGO). Because this strand now serves as the guide strand, these predominant strands would interact with target mRNAs through Watson-Crick base-pairing. However, if the opposite strand of the duplex is selected instead, then a completely new set of cognate mRNA targets would now be regulated by this miRNA. Because of this dynamic, the old nomenclature of calling a miRNA duplex a guide strand (i.e., dominant) or star strand (i.e., degraded strand) is outdated. Researchers now use the terms -5p or -3p

based on the location of the mature miRNA on the pre-miRNA stem loop (see Figure 2). Another reason for this new nomenclature, is that during normal cellular processes, (i.e. non cancerous tissue), deep sequencing studies have found that -5p mature miRNA sequences are expressed at times at almost similar levels to the -3p mature miRNA from the same miRNA stem loop precursor.

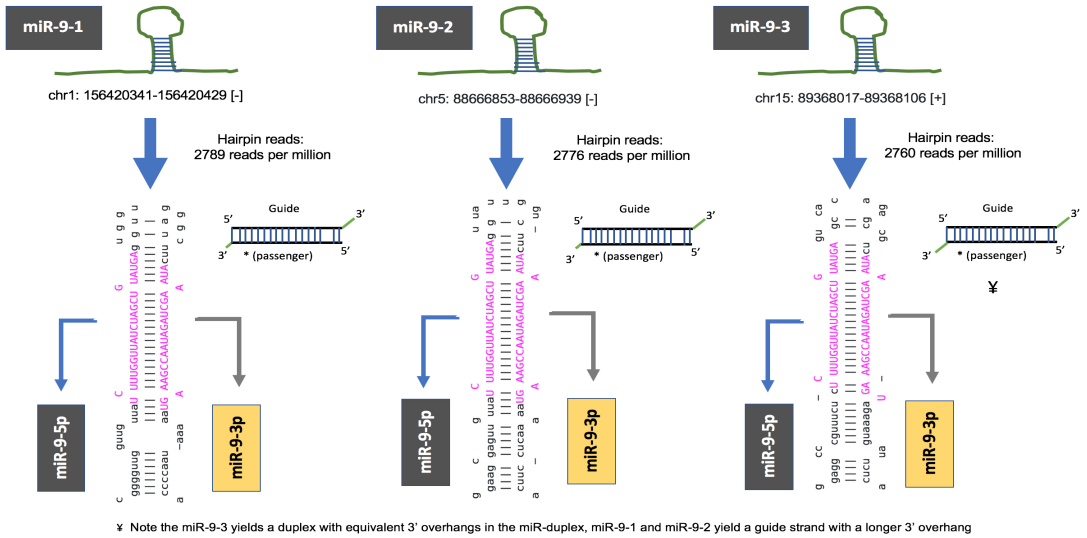


Figure 2. Genomic Loci of the miR-9 Host Genes and Isoform Characteristics.
This figure depicts the genomic loci from which miR-9 derives (green text). The miR-9 host gene is located on chromosomes 1, 5, and 15 respectively, with each miR-9 gene being processed into a pre-miRNA hairpin. The 85 nucleotide hairpin reads from deep sequencing are similar across all three isoforms. While all hairpins share similar configuration, the miR-9-3 isoform yields a miRNA duplex with equivalent 3' overhangs of 2 nucleotides each (shown in blue text and inset duplex diagram). This equivalency indicates balanced loading of miR-9 guide (-5p) and star (-3p) strands into the RISC complex, and therefore balanced loading into AGO2 complexes for mRNA targeting. A number of complex factor effects isoform abundance including DNA methylation, gene amplification, chromosomal abnormalities, and RNA trimming / editing.

These are fascinating discoveries and there may be some evolutionary explanations for how miRNA biogenesis and strand selection commences. First, the conservation of both miRNA precursors and certain mature miRNA strands across different species, sheds light on the evolutionary origins and functional significance of most miRNAs [116-118]. These mechanisms remain almost unchanged throughout thousands of years of evolution, and some miRNA sequences can be found in almost all species across nature in both plants and animals [118-120]. Additionally, the miRNA biogenesis machinery is also highly conserved across species, indicating miRNA regulatory pathways co-evolved with protein coding genes since the earliest known cellular organism. From a selfish genetic perspective, it is clear that mutations in the 3'UTR of genetic elements occur to avoid regulation by miRNAs, and in turn miRNA isoforms may be selected for, so as to keep these genetic elements in check. As a result of this complex interplay, emergence of families of miRNAs, miRNA duplications, and miRNA isoforms that share a similar sequence and function developed within the genome [121,122].

Finally, recent studies have found alternative miRNA biogenesis pathways that can yield new forms of miRNAs. Partly, this has to do with mutations of canonical miRNA processing genes such as DROSHA and DICER, which have been shown to play a role in developmental abnormalities and cancer [123-129]. Additionally, researchers have also identified specific RNA-binding proteins such as HnRNPA1, SMAD1 and SMAD5 to be important regulators of the miRNA biogenesis pathway, where in some cases the processing of A or G sequence rich pre-miRNAs becomes disrupted [130-136]. However, the most recent discovery is that alternative miRNA species called miRtrons can be formed during the mRNA splicing process due to the secondary structure of some intronic genetic sequences [42]. Importantly these short hairpin introns completely bypass DROSHA processing and can be loaded directly into DICER for further miRNA maturation. This phenomenon was first discovered in Drosophila and then found to occur throughout the animal kingdom, indicating this conserved pathway has evolved for the same reasons as canonical miRNA sequences, and indicates their crucial role in gene regulation [137-139]. To reiterate the importance of miRtrons, these hairpin structures are formed from short intronic sequences of protein-coding genes that are spliced during transcription forming lariat structures [54]. This means that miRtrons and the host protein coding gene may also exist in a co-evolutionary biochemical relationship. Also, because these hairpin structures bypass the DROSHA-DGCR8 complex, the short intronic lariats formed during the splicing must be debranched by the enzyme DBR1 in the nucleus, before being transported to the cytoplasm by Exportin-5. This indicates that there are still more biogenesis related genes that control miRNA production, and as such, represent another series of genetic elements that can become dysregulated during tumorigenesis. Finally, miRtrons are somewhat different than canonical miRNAs in that the mature miRNA sequence in miRtrons reside on the terminal loop of the pre-miRNA, extending from the 5' arm to the 3' stem (corresponding to guide and passenger strands) [55,56]. Therefore, there is almost no occurrence of strand selection during

DICER processing of a miRtron. Taken together, by understanding these nuanced biochemical processing steps, RNA researchers can uncover new species of RNA, initiate the development of novel biomarkers of disease, as well as to build innovative targets for therapeutic intervention.

4. Mirna Isoforms and Gene Duplication

In many cases, miRNA transcripts naturally contain sequence variations, called microRNA isoforms (isomiRs). These variations can be caused by inaccurate DROSHA or DICER cleavage or insertion mutations [140-142], but are usually not the result of trimming as depicted above. IsomiR abundance varies due to many factors, including cell type, tissue of origin, and other epigenetic factors. Although isomiRs are common, their broader biological significance is not fully understood; there are numerous databases that have compiled this miRNA sequence information, and data on isomiRs are limited due to the current methodological approaches utilized to accurately capture and quantify their abundance [140]. From the few studies that have investigated isomiR function, it seems isomiRs have a wide range of biological effects including the dysregulation of miRNA targeting, influencing strand selection, and altering the stability of miRNA bound RISC complexes.

IsomiRs can be classified in four ways in reference to the template sequence: a nucleotide variation, the length of the 5′ end of the miRNA sequence, a shorter or longer miRNA 3’ end, and/or a post-transcriptional RNA modification [57-61]. IsomiRs that contain the multiple characteristics noted above are identified as multi-variant isomiRs. From what little is known regarding isomiR formation, it is generally assumed that nucleotidyl transferases, which make edits to the RNA sequence during miRNA maturation, partly account for this variant formation [62]. Moreover, these edits tend to result in sequence variations at the 3′ end of the miRNA, which affect miRNA targeting efficacy rather than the target cognate mRNA repertoire. Researchers have found that there is a positive selection pressure for isomiR retention as these variants increase miRNA diversity and maintain a dynamic non-coding RNA regulatory network [143-149]. Clearly, isomiR expression and function is strongly related to their respective homologous miRNA, and may even function to prevent repressive genetic elements from silencing this respective miRNA gene loci [150]. The expression of isomiRs also varies in certain cells and tissues, resulting in many different physiological outcomes, such as variant accumulation in certain types of cancer.

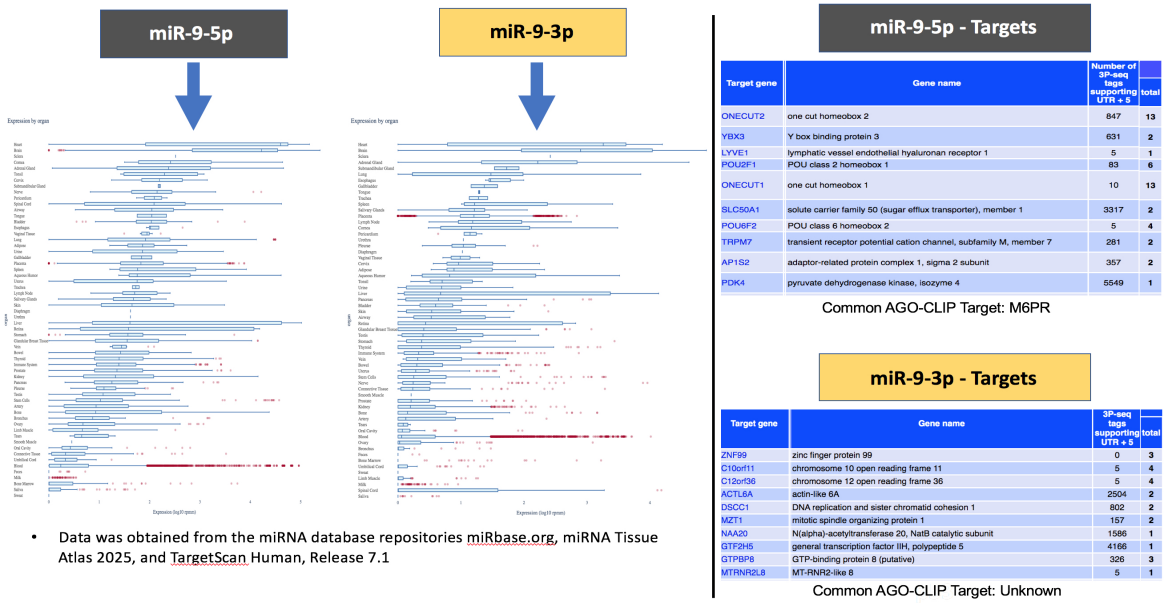
A great example of a canonical miRNA that has a number of iso-variants is miR-9. miR-9 plays a pivotal role in neural development by targeting key transcription factors such as, HES/HER and ERK1/2 [151]. In zebrafish, miR-9 expression and activity modulates HER6 transcriptional dynamics, maintaining a low or noisy oscillatory range of gene expression. When neural progenitors remain in an undifferentiated state, miR-9 levels tend to be low. Yet, during differentiation miR-9 levels rapidly increase causing a dramatic decrease in HER6 activity allowing for late-stage neuronal differentiation [152,153]. This process depends on the precise levels of miR-9, as deviations in expression can disrupt HES1/HER6 oscillations and the timing of neuronal differentiation in Zebrafish. Notably, vertebrates possess multiple miR-9 loci, each maintaining a respective temporal and spatial gene expression pattern (see Figure 2) [154-160]. Given what is known regarding the quantitative mechanisms that maintain miR-9 activity during Zebrafish neuronal development, one could argue this regulatory pathway is conserved across species including mammals. Specifically, researchers are now investigating how early and late pri-miR-9 transcript isoforms accumulate within the cell, resulting in the enhanced mature miR-9 levels essential for HER6 downregulation and late-stage neuronal differentiation [161-167]. These studies underscore how intricately nuanced a non-coding RNA regulatory framework can be. Understanding both the qualitative and quantitative contributions of pri-miR-9 isoforms during neural development is crucial in elucidating which molecular pathways found to be disrupted in cancer are miR-9 dependent.

Some investigators have assessed the 3D structural characteristics of pri-miRNAs to determine how certain RNA motifs are recognized by DROSHA, and what moieties may influence the precise cleavage of the pre-miRNA stem loop [168-170]. Researchers argue that the flexibility within the lower-stem portion of the hairpin within the pri-miRNA creates a base pairing distortion influencing the accuracy of nucleotide cleavage. This structural ambiguity is observed with miR-9 itself, given certain miR-9 paralogs have non-equivalent 3’ overhangs post DICER cleavage (see Figure 2). Because of this ambiguity, strand separation of the miRNA duplex is a much more stochastic process. This indicates that both DROSHA and DICER require precision for proper catalytic function, and that any sequence variation can destabilize the fidelity of these RNaseIII enzymes, adding complexity to the regulation of miRNA biogenesis [168-170].

5. The Cell Context Expression of Mirnas

Researchers now understand that the processing of miRNAs, including isomiR variants, is a tissue-specific process, which has profound and significant biological implications. The seemingly ubiquitous expression pattern of miR-9 and miR-9 variants in the nervous system seemingly argues against this notion [171]. However, miR-9 variants are somewhat differentially abundant in other tissue types (see Figure 3). Importantly, in pathological states, the presence of certain miRNA isoforms predominant for a number of reasons. miR-9-5p for instance is clearly present in low-grade glioma datasets, underscoring the potential for this miRNA to be a *bona-fide* cancer-specific biomarker. However, in AML, miR-9-3p is present at much higher levels and is directly attributable to leukemogenesis. Again the reason for the biased miRNA isoform expression in each of these tissues is not well understood, however some

studies indicate the occurrence of "miRNA-editing-hotspots," in cancerous tissues, with RNA editing levels at rates above 5% in some tumor types [172-178]. Furthermore, from an analysis of 8,595 TCGA samples across 20 tumor types, miRNA-editing hotspots correlated with the expression of known oncogenes, emphasizing the relevance of miRNA modifications during the process of tumorigenesis.



miR-9-5p - Targets

Target gene	Gene name	Number of 3P-seq tags supporting UTR + 5	total
ONECUT2	one cut homeobox 2	847	13
YBX3	Y box binding protein 3	631	2
LYVE1	lymphatic vessel endothelial hyaluronan receptor 1	5	1
POU2F1	POU class 2 homeobox 1	83	6
ONECUT1	one cut homeobox 1	10	13
SLC50A1	solute carrier family 50 (sugar efflux transporter), member 1	3317	2
POU6F2	POU class 6 homeobox 2	5	4
TRPM7	transient receptor potential cation channel, subfamily M, member 7	281	2
AP1S2	adaptor-related protein complex 1, sigma 2 subunit	357	2
PDK4	pyruvate dehydrogenase kinase, isozyme 4	5549	1

Common AGO-CLIP Target: M6PR

miR-9-3p - Targets

Target gene	Gene name	3P-seq tags supporting UTR + 5	total
ZNF99	zinc finger protein 99	0	3
C10orf11	chromosome 10 open reading frame 11	5	4
C12orf36	chromosome 12 open reading frame 36	5	4
ACTL6A	actin-like 6A	2504	2
DSCC1	DNA replication and sister chromatid cohesion 1	802	2
MZT1	mitotic spindle organizing protein 1	157	2
NAA20	N(alpha)-acetyltransferase 20, NatB catalytic subunit	1586	1
GTF2H5	general transcription factor IIH, polypeptide 5	4166	1
GTPBP8	GTP-binding protein 8 (putative)	326	3
MTRNR2L8	MT-RNR2-like 8	5	1

Common AGO-CLIP Target: Unknown

Figure 3. Normal Tissue Specific miR-9 Expression Patterns and Top Ten mRNA Targets.
This figure depicts a more balanced way of assessing miRNA isoform strand abundance via tissue specific sequencing, as opposed to the original whole organism cloning analysis. Here we find miR-9-5p and miR-9-3p are both abundantly expressed in heart, brain, and lung tissue. miR-9-3p is expressed in general slightly less than miR-9-5p, yet the presence of both isoforms in these tissues supports the notion of equivalent loading into AGO complexes. The top ten predicted targets of miR-9-5p and miR-9-3p are also shown on the right panel, with a known miR-9-5p target M6PR shown to be biochemically linked with AGO2 via CLIP-seq data. These data support that both miR-9 strands are contributing to stochastic gene regulation that can promote or suppress tumorigenesis in epithelia tissue.

Together, these findings highlight the intricate regulatory processes that control miRNA abundance, which includes RNA-editing, trimming, and strand selection, all of which contribute to the miRNA functional diversity within the cell. These unique isoforms and imperfect duplexing of miR-9 pre-miRNAs further complicate the process of strand selection between miR-9-5p and miR-9-3p, and potentially explains the differential abundance of these isoforms in specific tissues. These insights into the processing and editing of miR-9 and related miRNA isoforms not only enhances our understanding of miRNA biogenesis but also reveals new genetic regulatory loops that are actively occurring during normal development, as well as disease pathogenesis. Elucidating these regulatory loops will inform clinical scientists as to how to develop effective therapeutics involving non-coding RNA agents.

6. The MiR-9 Family

As mentioned above, miR-9 is a unique miRNA molecule expressed in a specific group of inactive radial glial cells. miR-9 is one of the most expressed miRNAs within the developing brains of adult humans (see Figure 3), and plays a crucial role in maintaining cell quiescence by regulating gene expression signaling pathways such as NOTCH and FOXG1 [179,180]. As is typical of most miRNAs, miR-9 functions as a developmental lock operating as a protective feature allowing for the proper proliferation and self renewal of neural progenitor cells. The regulators of miR-9 would then serve as the key to initiate a repertoire of cellular functions during neuronal specialization. The diversity of miRNA function is quite striking as miR-9 controls neuronal migration, differentiation, proliferation and apoptosis, while the loss of miR-9 plays a central role in neurodegenerative disease [181].

miR-9 is also a highly conserved miRNA and studies in model organisms such as *Xenopus laevis*, *Danio rerio* (zebrafish), and *Mus musculus* (mice) indicated that miR-9 is essential for cell fate transitions during neurogenesis by post-transcriptionally regulating the transcription factors FOXG1 and TLX1 [182-185]. Interestingly, during zebrafish neurogenesis, the amount of mature miR-9 increases in a sharp, stepwise manner, driven by the sequential expression of seven distinct pri-miR-9 transcripts. Furthermore, the expression of late-onset pri-miR-9-1 occurs despite the presence of early-onset pri-miR-9-4 and -9-5 transcripts in individual cells, underscoring the importance of a redundant positive feed-forward expression program vital for zebrafish neurogenesis [167]. Notably, CRISPR/Cas9-mediated disruption of pri-miR-9-1 locus impairs the developmental increase of mature miR-9, resulting in the inability of neurons to downregulate HER6 and subsequently causes a reduction in late stage neuronal differentiation. This knockout model emphasizes the precise temporal and spatial regulation of miR-9 as a fundamental regulatory mechanism during neurogenesis [186]. Beyond the role of miR-9 in neural progenitors,

miR-9 controls dendritic cell growth and synaptic transmission *in vivo*. Downregulation of the transcriptional repressor REST, mediated by miR-9, is necessary for proper development and growth of neuronal dendrites [187]. miR-9 is also linked with spinal cord development in vertebrates by controlling FOXP1 and ONECUT1. This suggests that miR-9 is critically important in motor neuron development [188,189]. However, given miR-9 is expressed in the forebrain, midbrain, and hindbrain of developing animals, it is clear that the spatiotemporal expression and activity of miR-9 plays an important role in many neurological disorders, as well as in supporting normal neurocognitive phenotypes. Overall, miR-9 regulates multiple anatomical-specific genes within the brain in a precise spatiotemporal manner during vertebrate neural development.

7. The Context Dependent Regulatory Action of MiR-9 in Cancer

The context-dependent regulatory action of miR-9 in cancer highlights a dual role for miR-9, as both a tumor suppressor and an oncogene. The actions of miR-9 in supporting or repressing tumorigenesis really depends upon the cancer type as well as the molecular environment, meaning the abundance of the cognate target cellular mRNA profile [190]. miR-9 exerts its effects through diverse mechanisms, targeting key pathways involved in cellular proliferation, survival, migration, and invasion (see Table 1). For instance, in medulloblastoma miR-9 functions predominantly as a tumor suppressor by downregulating the oncogenic transcription factor TLX1, while simultaneously modulating the NOTCH/STAT signaling pathway [191-194]. In glioma, however, miR-9 often operates as an oncogene, promoting angiogenesis and tumor growth through targets such as COL18A1 and PTCH1 [195]. Similarly, in breast cancer, miR-9 has seemingly conspicuous roles depending upon the cell types that express the miRNA. For instance, miR-9 can suppress growth in 17-β estradiol receptor-positive tumors but may drive a more aggressive triple-negative breast cancer subtype by promoting MAPK signalling. This seemingly dual functionality extends to other cancers, including gastric, prostate, and blood tumors, where miR-9 regulates ether known oncogenic or tumor-suppressive targets such as TNFAIP8L3, StarD13, and NF-κB signaling [196-198]. The intricate regulation of miR-9 by genetic, epigenetic, and transcriptional co-factors underscores miR-9 as a potential biomarker and a therapeutic target across many cancers. Further research is required to unravel the complex, and cellular context-specific roles of miR-9 within each cancer subtype.

7.1 Medulloblastoma

In the context of medulloblastoma, miR-9 has garnered significant attention as a potential tumor suppressor (see Table 1). Experimental evidence from *in vitro* and *in vivo* studies has demonstrated that miR-9 negatively regulates critical oncogenic pathways and genes associated with medulloblastoma pathogenesis [199,200]. For instance, miR-9 has been shown to target and downregulate TLX1, a transcription factor implicated in medulloblastoma pathophysiology, thereby attenuating certain anti-tumorigenic effects. Additionally, miR-9 has been reported to modulate other key mediators of cellular proliferation and survival, such as NOTCH1 and STAT signaling, potentially impacting tumor growth and therapy resistance [201,202]. The dysregulation of miR-9 in medulloblastoma may be due to various mechanisms, including genetic alterations, epigenetic modifications, and dysregulation of upstream co-regulatory transcription factors.

In support of miR-9 operating as a tumor suppressor, aberrant DNA methylation and histone modifications near the genomic loci of miR-9 have been observed in medulloblastoma samples, leading to the suppression of miR-9 activity. Moreover, transcriptional regulation mediated by oncogenic transcription factors can also influence miR-9 expression levels, thus contributing to tumor pathogenesis [203,233]. Beyond the role of miR-9 as a tumor suppressor, miR-9 has shown promise as a potential diagnostic and prognostic biomarker for medulloblastoma. Studies have investigated the differential expression of miR-9 in tumor tissues as compared to normal brain tissue, as well as the correlation with clinicopathological features and patient outcomes [203,233]. These studies have shown that miR-9 inversely associates with tumor size, clinical stage, and distance metastasis. Therefore, the further characterization of miR-9 expression patterns in medulloblastoma could aid in refining molecular subtyping and stratification criterion for the disease. Moreover, these findings have implications for developing novel treatment approaches for those medulloblastoma patients with poorer prognosis, such as group 3 and 4 tumor types.

Table 1. Notations of the Various miR-9 Isoforms Involved in Tumorigenesis.

Cancer Type	Tumor Suppressor/Oncogene	Overview	miR-9 Isoform	References
Medulloblastoma	Tumor Suppressor	Downregulates TLX1 Modulates NOTCH1	hsa-miR-9-5p	[199-202]
		Modulates STAT signaling Modulates DNA methylation	hsa-miR-9-5p	
		Downregulated in MB	hsa-miR-9-5p	
Glioma	Oncogene	Promotes angiogenesis Downregulates COL18A1	hsa-miR-9-5p	[195,203-206]
		Downregulates PTCH1 Modulates HIF-1α/VEGF Upregulated by MYC	hsa-miR-9-5p	
		Upregulated in Glio	hsa-miR-9-5p	
Breast Cancer	Both	Downregulates ERa+ Modulates MAPK	hsa-miR-9-5p	[207-212]
		Promotes tamoxifen-resistance Promotes TNBC phenotype	hsa-miR-9-5p	
		miR-9* suppresses TNBC proliferation and migration	hsa-miR-9-3p	
		miR-9* targets ITGB1 miR-9* targets MEK	hsa-miR-9-3p	
Cervical Cancer	Both	Upregulated in CC/SCC	hsa-miR-9-5p	[213-217]
		Modulates HPV16/18-related	hsa-miR-9-5p	
		Downregulates TWIST1 Downregulates FOXO3	hsa-miR-9-3p	
		Tumor suppressor in AC	hsa-miR-9 ?	
Prostate Cancer	Both	Downregulates StarD13 Downregulates NUMB	hsa-miR-9-5p	[218-221]
		Promotes stemness Promotes proliferation	hsa-miR-9-5p	
		Tumor suppressor functions	hsa-miR-9-3p	
		Downregulates MEKK3 Restores E-cadherin and MET.	hsa-miR-9-3p	
Gastric Cancer	Both	Inhibits migration and invasion	hsa-miR-9-5p	[222-227]
		Targets TNFAIP8L3 & RAB34	hsa-miR-9-5p	
		Upregulates MYH9 due to HULC sponges	hsa-miR-9-3p	
		Targets CDX2 and CDH1 Promotes tumor progression	hsa-miR-9-3p	
Chronic Lymphocytic Leukemia (CLL)	Tumor Suppressor	Downregulated by hypermethylation	hsa-miR-9-5p	[228-230]
		5-AzadC restores miR-9 expression	hsa-miR-9-5p	
		Targets NF-κB1 Pro-tumor suppressor effects	hsa-miR-9-5p	
Acute Myeloid Leukemia (AML)	Both	Suppresses CXCR4 Promotes G1 arrest and apoptosis	hsa-miR-9-5p	[231,232]
		In MLL/AML miR-9, promotes tumor progression	hsa-miR-9-3p	
		Targets RHOH and RYBP	hsa-miR-9-3p	

* In some cancers it is not clear if miR-9-5p or miR-9-3p drive tumorigenesis as miRNA overexpression stem loop methods as well as gene knockout methods modulate the levels of both miRNA isoforms.

7.2 Glioma

Glioma is characterized by cancerous glial cell growth in the brain or spinal cord, and develops due to unabated angiogenesis and tumorigenesis. The underlying factors that trigger or inhibit these molecular processes is mostly unknown. miR-9 regulates biological processes and cellular pathways that ultimately supports glioma tumorigenesis (see Table 1). Some researchers have indicated glioma as a miR-9 type tumor given the elevated levels of miR-9 that are found in most all glioblastoma tumor tissue. Furthermore, miR-9 promotes the enhanced proliferation, migration, and invasion of glioma cells by targeting COL18A1, THBS2, PTCH1 and PHD3 [68,184,195]. Interestingly, miR-9 can be transported within exosomes and absorbed by vascular endothelial cells, increasing tumor angiogenesis, one of the hallmarks of aggressive drug-resistant glioblastoma.

The mechanisms supporting persistent miR-9 expression in glioblastoma may be related to the structure of the miR-9 genes. Studies have shown that MYC and OCT4 can bind to the miR-9 promoter and transcriptionally activate miR-9 in an uncontrolled manner [204,205]. This mechanism would support the uncontrolled proliferation and expansion of neural progenitor cells. According to the two hit model of tumorigenesis, if a second mutation occurred within this expanded stem cell pool, the formation of an aggressive undifferentiated glioblastoma could easily develop. Therefore, it should be appreciated that miRNAs may be indirect drivers of tumorigenesis by facilitating an environment that favors tumorigenic development and progression.

What is less understood is how each miR-9 isoform contributes to tumorigenesis. In the case of both medulloblastoma and glioblastoma it is clear miR-9-5p is driving a tumor suppressive and oncogenic phenotype, respectively. This indicates that the cognate target mRNAs expressed in each cell type is an important determinate of miR-9 function in these cancers. Of course many researchers have not fully investigated the functional consequence of miR-9-3p in these tumors.

Despite these limitations, the use of miR-9 as a therapeutic for glioma is becoming a reality due to the fundamental role of miR-9 in glioma pathogenesis. Researchers know that miR-9 directly targets the 3'-UTRs of COL18A1, THBS2, PTCH1, and PHD3, degrading these mRNAs and disrupting HIF-1α/VEGF signaling [195]. This regulatory effect is crucial in developing miR-9 as a therapeutic given angiogenesis is a major contributing factor supporting malignant glioblastoma [68]. What has limited miR-9 as a therapy for various forms of brain cancer involves the effective delivery of the therapeutic oligonucleotide to the brain tissue, and importantly ensuring that the RNA entity effectively inhibits only the pro-tumorigenic functions of the miRNA. Therefore newer oligonucleotides have been developed to only express one miRNA-strand, an anti-miR that effectively blocks miRNA activity, or an oligonucleotide that only disrupts one miRNA-mRNA pairing. These findings highlight miR-9 as a potential target for glioma diagnosis and therapy.

7.3 Breast Cancer

miR-9 has a pivotal role in breast cancer development, as the targets of miR-9 are clearly cell context dependent. Similar to the neuronal system, and perhaps more prevalent, miR-9 has been shown to target ERα [234-238]. Similar to miR-206[23], the regulation of ERα by miR-9 indicates that miR-9 not only functions as a tumor suppressor in 17-β-estradiol addicted hormone positive breast cancer, but may also serve as a promoter of an oncogenic event by promoting the formation of a triple negative breast cancer cell [180]. This process seems paradoxical, yet one must understand that very few proliferative cells of the mammary gland are in fact ERα+. A majority of the dormant mammary stem cell pool are ERα-. Similar to glioblastoma, if miR-9 expression supports stem cell expansion in these cell types, then a second oncogenic mutation would result in the formation of an ERα-, hormone independent breast cancer. In support of this hypothesis, the forced expression of miR-9 in ER+ MCF-7 cells, results in an initial decrease in cell proliferation, however prolonged exposure to miR-9 results in the emergence of MCF-7 tamoxifen resistant cells [207-209,234]. Furthermore, these new cells also take on the characteristics of a more stem/precursor cell phenotype. Therefore, we suspect the best utilization of a miR-9 therapy would be an anti-miR-9 in patients with tamoxifen resistance tumors.

Despite this assumption above, a major question remains as to what form of miR-9 therapy should be implemented (see Table 1). Returning to the concern of miR-9 isoforms, in the experiments above, a lentivirus containing a miR-9 precursor stem loop was utilized. This means that both miR-9-5p and miR-9-3p strands are being expressed at abundant levels in these MCF-7 cells. Many researchers do not assess the targets of miR-9-3p in these studies, only the associated loss of ERα due to miR-9-5p targeting [210,239]. Therefore, miR-9-3p could serve as a substrate for a miRNA-based therapy in tamoxifen resistant breast cancer by sponging and blocking miR-9-5p function [239].

Interestingly, some have reported that the entire genetic miR-9 locus is hypermethylated in TNBC tumors given miR-9 can target the MAPK pathway. It is known that TNBC cells rely on MAPK for growth and proliferation [211,212]. Yet what does this mean for the expression of miR-9-5p and miR-3p isoforms? Further research shows that miR-9 is expressed from three distinct genetic loci, and therefore this reported hypermethylation may not be a complete process (see Figure 2). To circumvent the concern of a miR-9 mimic therapy supporting tamoxifen resistance in ERα+ tumors, the use of a miR-9-3p therapeutic could have superior potential in the clinic. In fact, miR-9-3p appears to not have pro-growth roles in TNBC, and miR-9-3p has been shown to suppress growth and proliferation of TNBC cells in combination with a MEK inhibitor. Also, by regulating the ITGB1, miR-9-3p also appears to influence and abate TNBC cellular migration, a key feature of metastatic ERα- breast cancer.

The data suggest that the biological processes of miRNAs are seemingly far more complex than originally

anticipated. However, the methods utilized to study miRNA-mRNA regulation *in vitro* [236], does not always translate to the effects mediated by a chemically modified single strand oligonucleotide. In fact, the notion of using a stable miRNA isoform to sponge culprit miRNAs during tumorigenesis may be a more practical therapeutic approach rather than standard miRNA therapeutic strategy that brings about the concern of modulating numerous mRNA, causing off-target effects.

7.4 Cervical Cancer

Cervical cancer is the fourth most common cancer among women worldwide, with over 570,000 new cases and more than 311,000 deaths reported annually [213]. A number of miRNAs have been associated with cervical cancer progression and drug response, and shown to play critical roles in regulating gene expression and influencing disease outcomes. Specifically, miR-9, along with miR-21 and miR-155, are significantly overexpressed in cervical cancer tissues compared to normal tissues (see Table 1) [213,214]. During cervical cancer development, deregulation of miRNAs, including miR-9-5p, contributed to aberrant gene expression. Interestingly, miR-9-5p associates with a high-risk human papillomavirus (hr-HPV)-type-dependent expression pattern, and functions as an oncogene in squamous cervical carcinomas, while potentially promoting a tumor suppressor phenotype in cervical adenocarcinomas. This dual functional role of miR-9-5p underscores the complexity of miR-9-5p regulation and highlights the potential of certain miR-9 variants as diagnostic and therapeutic targets in cervical cancer.

Again, both miR-9-5p and miR-9-3p isoforms are expressed in normal cervical tissue (see Figure 3), however, most researchers only focus on the presumed predominant miR-9-5p isoform. This could be due to the fact that persistent infection with hrHPV, particularly HPV16 and HPV18, results in extremely elevated levels of miR-9-5p [213-217,240]. While HPV is one of the primary causes of cervical cancer, many other cancer subtypes can emerge absent of HPV infection. Given we know miR-9 functions in different capacities given the pathological subtypes of cervical cancer, (i.e., squamous cell carcinoma versus adenocarcinoma), it would also be important to know if certain miR-9 variants are correlated or anti-correlated with HPV. Given squamous cell carcinomas account for approximately 80% of cervical cancer cases [213,241], understanding the role of miR-9 in this cellular context is quite important (see Table 1). This is mostly due to the hyperfocus on elucidating the molecular underpinnings of epithelial cervical adenocarcinomas which is driven by HPV infection. In support of this, in squammous cancers of the head and neck it seems miR-9 functions as a tumor suppressor by stalling cell cycle. Therefore one cannot simply assume that miR-9 operates as a tumor suppressor or oncogene solely based on the histological categorization of the tumor. Moreover, an in depth study of all known miR-9 variants should be performed in future tumor expression studies so as to fully understand the dominant isoform variants that may be driving the tumorigenic process through specific and undiscovered regulation of cognate mRNA gene targets.

7.5 Prostate Cancer

Prostate cancer is one of the most common types of cancer in men, and a leading cause of cancer mortality among men [242,243]. miR-9 expression has been shown to impact prostate cancer biology and progression (see Table 1). More specifically in DU145 and PC-3 cell lines, miR-9 promotes cell proliferation, invasion, and epithelial to mesenchymal transition (EMT), promoting cancer progression [218-220]. The pro-onogenic effects of miR-9 are due to the direct targeting of StarD13, a known tumor suppressor gene. Furthermore, in prostate cancer cells, miR-9 and StarD13 expression levels are anti-correlated [198], further supporting the notion that StarD13 is one of the main targets of miR-9.

A common theme in epithelial tumors is the loss of cellular polarity and the acquisition of a migratory and mesenchymal phenotype. Interestingly, the restoration of StarD13 by miR-9 inhibition results in upregulation of E-cadherin, a critical tumor suppressor protein responsible for maintaining cell-cell adhesion and cellularity polarity. This approach also results in increased N-cadherin and a decrease of vimentin expression, indicating a complete reversal of the EMT process. Therefore inhibition of miR-9 may very well be a valid therapeutic strategy to stall prostate cancer progression.

Another mechanism of action by miR-9 in prostate cancer involves CD44. Specifically, some studies have shown that miR-9 is significantly upregulated in CD44+ prostate cancer stem cells (PCSC) by suppressing the tumor suppressor NUMB [198]. Although it is unclear which miR-9 isoforms play a role in this process, it is clear that forced expression of miR-9 (via lentivirus continuing a miR-9 stem loop), promoted stemness in PCSCs, supported increased sphere formation and clonogenic capabilities, and ultimately promoted a drug resistant phenotype in tissue culture [244-246]. miR-9 can also reduce apoptosis by lowering Caspase-3 expression and supporting proliferation elements such as Ki67 [198].

One challenge in the prostate cancer field is that some tumors have a differential dependence on androgen receptors to support growth and proliferation. However, this steroid hormone response is not as robust as it is in the mammary gland, resulting in more complex molecular heterogeneity with respect to cancer subtyping. We raise this point, because a different study indicated that miR-9 is significantly down-regulated in prostate cancer cell lines as compared to normal prostate cells [221]. Furthermore, miR-9 has been shown to target MEKK3, an oncogenic kinase that promotes cellular proliferation, migration, invasion, and EMT [221,247]. However, none of these studies have carefully assessed the abundance or activity of miR-9 variants in response to testosterone levels. It may very well be that certain miR-9 variants predominate under conditions of low androgen levels, which would result in a different repertoire of dysregulated mRNA targets that may either promote or inhibit tumorigenesis. Only in depth genetic

and mechanistic genetic studies can address these differential cellular mechanisms.

7.6 Gastric and Liver Cancers

Gastric cancer (GC) is a leading cause of cancer deaths in humans [248]. Studies show that miR-9 expression is significantly reduced in both GC cells and the plasma of GC patients as compared to normal healthy controls [222]. More specifically, miR-9 inhibits the migration and invasion of GC cells by targeting TNFAIP8L3. Furthermore, miR-9 binds to the 3' UTR of TNFAIP8L3 reducing its expression, and thereby reducing the aggressiveness of GC cells, as confirmed by transwell and wound-healing assays *in vitro* [196]. These studies suggest miR-9 is a putative tumor suppressor in gastric cancers. Additionally, it has been shown that miR-9 targets genes such as RAB34, cyclin D1, and Est1, all of which are involved in tumor progression and metastasis, and inhibit NF-κB1, reducing tumor growth and invasion [223,224]. Finally, miR-9 downregulation also shows strong correlations to clinicopathological features of tumor progression in patients [225].

Upon further investigation it appears miR-9-5p is the miR-9 isoform driving this phenotype, with almost no data on the miR-9-3p variant. This is relevant, as miR miR-9-3p is expressed in stomach and liver tissues (see Figure 3), and therefore may have biological activity. In support of this, miR-9-3p appears to be elevated in liver cancer and targets the non-coding RNA HULC. This is a fascinating story because the elevated expression of miR-9 is not directly linked with the activity of miR-9. In fact, miR-9 directly targets myosin heavy chain 9 (MYH9), a gene linked to cancer metastasis. HULC acts as a sponge for miR-9, reducing its availability and consequently allowing for MYH9 to be expressed. This suggests that silencing HULC can inhibit gastric cancer progression by controlling the miR-9/MYH9 axis, opening a potential therapeutic window for this pathway by targeting HULC [226,249,250]. However, what has not been addressed in this study is if miR-9-3p becomes engaged with HULC, is there an accumulation of miR-9-5p variants? Furthermore, what are the regulatory effects miR-9 variants have on the tumorigenic process.

Another example of differential miR-9 activity in liver cancer involves the miR-9, CDX2 story. Here, miR-9 targets and suppresses the tumor suppressor genes CDX2 and CDH1 (i.e., E-cadherin) resulting in pro-oncogenic properties [251]. The use of LNA-modified AMOs, designed to target and bind to miR-9-5p, resulted in the elevated expression of CDH1 mRNA, and restoration of E-cadherin levels [252]. These findings again, suggest promising strategies for the direct therapeutic targeting of a miRNA variant. Here the targeting of miR-9-5p with LNA's blocks the function of miR-9-5p. Additionally, miR-9 could be used as a diagnostic tool for the management, identification, and prognosis of gastric cancer. Taken together, given the complex role of miR-9 in gastric cancer further research is needed to parse out the dual functionality of miR-9.

7.7 Leukemia

As discussed earlier, miR-9 was originally identified as a tumor suppressor in chronic lymphocytic leukemia (CLL). In fact, one of the miR-9 host genes is extensively hypermethylated in approximately 17% of patient samples [253]. This epigenetic silencing leads to decreased miR-9 expression, contributing to tumor progression. miR-9 targets and suppresses NFκB1, a key activator of tumorigenesis through the promotion of cell proliferation and activation of anti-apoptotic genes (see Table 1). By downregulating NFκB1, miR-9 limits tumor cell survival and reinforces its role as a tumor suppressor in CLL [228-230]. To further support this, treating CLL cells with 5-Aza-2′-deoxycytidine (5-AzadC), a DNA de-methylating agent, restored miR-9 expression resulting in reduced NFκB1 activity, and thus protumor-suppressive effects. While 5-AzadC is considered a crude therapeutic compound as it would result in many genes becoming de-methylated and re-activated, another approach may be to develop synthetic RNA oligonucleotide that could restore the function miR-9-5p variants, showcasing the therapeutic potential for miR-9 in CLL.

In the case of acute myeloid leukemia (AML), the expression of miR-9 is significantly downregulated, and the loss of miR-9 expression correlates with lower survival rates in AML patients [231,232]. The mechanism of action for miR-9 occurs through the direct binding and targeting of the CXCR4 3' UTR mRNA, resulting in reduced CXCR4 expression. CXCR4 is a crucial gene that supports AML cellular proliferation via extending the G1 phase of mitosis. CXCR4 also supports chemokine induced cell migration, as blood cells home to certain tissue regions based on the gradient of chemokine expression. The re-expression of miR-9 in AML results in G1 phase arrest and apoptosis, as well as stalled cellular migration. These findings highlight that the miR-9/CXCR4 axis function as a promising therapeutic target in AML.

What is not well understood though, is whether miR-9-5p or miR-9-3p would be the best candidate for a therapeutic in AML. This is because re-expression of the silenced miR-9-5p in AML cells targets the CXCR4 gene resulting in anti-tumorigenic phenotypes. However the silencing of miR-9 genetic loci in AML also means that miR-9-3p levels would also be reduced. However, there is no study that really assesses the levels of miR-9-3p, and/or determines whether miR-9-3p becomes elevated in AML. This is an important question, as there is evidence that miR-9-3p is overexpressed in MLL-rearranged type AML when compared to both normal controls and non-MLL-rearranged AML [190]. MLL-AF9 is probably one of the most common fusion proteins generated by chromosomal abnormalities and is the oncogenic driver in AML patients. For some unknown reason, miR-9-3p is expressed at elevated levels in MLL-AF9. miR-9 promotes leukemogenesis by suppressing expression of the transcription factors RHOH and RYBP, which are critical for normal hematopoiesis and apoptosis regulation [254]. Researchers have yet to

understand why miR-9-5p has a diminished role in MLL-AF9 AML. Perhaps there are differential levels of RNA editing that affect DICER recognition of the miRNA duplex. Taken together, these findings highlight that miR-9-3p is certainly a potential therapeutic target in MLL-AF9, while miR-9-5p may be a target in non-MLL-rearanged AML. Yet, more in-depth studies are required to determine which miR-9 isoforms predominate in these hematopoietic cancers. With this information, potent miR-9-5p or miR-9-3p decoy oligonucleotides could be developed so as to offer a promising treatment avenue for patients with aggressive leukemia subtypes that confer poor prognosis.

8. Therapeutic Delivery of Synthetically Modified MiR-9

Over the past decade the biochemistry supporting development of RNA-based therapeutics has advanced on many levels. These next-generation miRNA therapeutics still function by responding to complementary sequences in either a target miRNAs or candidate mRNAs. This is due to the fact that a number of modifications can be made to the backbone of the RNA oligonucleotide that facilitate cellular uptake, enhanced target duplex stability, and even result in the recruitment of specific RNA binding proteins to the miRNA targeting loci. In addition to these modifications, new delivery systems that involve miRNA packaging into exosomes and/or nanoparticles have paved the way for exponentially more intuitive RNA drug design for the oncological field.

As an example, in the case of brain cancer, miRNA therapeutics have been developed to function by re-expressing the lost activity of tumor suppressive miRNAs, and in other cases to sponge/decoy oncomiRs [255-259]. The development of these miRNA therapeutics has recently focused on enhancing the *in vivo* stability of RNA molecules and in designing precise delivery systems for tissue-specific release with minimal toxicity [260-264]. The limitations of these pre-clinical studies have been centered around the issue of effective delivery of these compounds to the anatomical regions where these tumor cells reside, as well as the concern of off-target effects of the RNA-oligonucleotide. Surprisingly, the common criticism of RNA therapy is that these oligonucleotides could bind other mRNA targets which could contribute to some side effects in patients. However, small drug kinase inhibitors are known to be promiscuous as well, given these drugs target the conserved ATPase pocket shared amongst almost all known protein kinases.

Another approach has therefore been to use the knowledge regarding a particular miRNA pathway and to then develop RNA oligonucleotides that interact with culprit mRNAs that operate as tumor suppressors or oncoproteins. This has been most apparent in the field of oncology. Specifically, in brain cancer, certain nanoformulations of a synthetic short-interfering RNA (siRNA) to TGF-β (a known target of certain miRNAs) have been developed to cross a synthetic blood–brain barrier in certain lab models [265-268]. From these studies it appears the gene silencing efficiency of si-TGF-β enhances the cytotoxicity of temozolomide (TMZ) in glioblastoma cell lines. Additionally, Xu *et al.* developed a pH-responsive nanoplatform that delivers an siRNA directed against BRD4. First BRD4 is a well known miR-9 target, and secondly an siRNA directed to BRD4 has potent anti-tumorigenic effects by altering the local tumor microenvironment. The unique strategy of the si-BRD4 platform is that this nanoplatform consists of a PHMEMA core which responds to changes in pH present in the acidic tumor microenvironment, while containing a PEGylated outer shell to protect and maintain the RNA compound while being circulated within the blood. This PHMEMA technology therefore, results in enhanced therapeutic payloads being delivered to the anatomic locations where the tumor cells of interest reside.

These studies highlight challenges such as efficient RNA delivery, miRNA stability, and deleterious immune responses. Yet, given the evidence supporting the effectiveness and safety of clinically approved mRNA vaccines (i.e., COVID-19), coupled with growing interest in mRNA-based therapeutics, mRNA technology is poised to become one of the major pillars of cancer drug development. Because miRNA/mRNA therapeutics deliver synthetic RNA into cells to regulate protein expression, or therapeutically re-express proteins of interest, respectively, these compounds are actually a safer alternative to DNA-based therapies that integrate into the host genome(i.e., concern with viral based delivery systems). Clinical scientists have actually developed *in vitro* transcribed mRNA-based therapeutics for cancer treatment, which include the characteristics of the various synthetic mRNAs as well the packaging systems for efficient mRNA delivery [269-274]. Taken together, there are still current challenges remaining in the field, such as the need for improved delivery mechanisms and management of the patient immune response. Despite these hurdles, there is optimism about the future prospects of miRNA/mRNA-based therapies. It is anticipated that many promising mRNA-based treatments currently under investigation will eventually translate into clinical applications, ultimately benefiting patients by providing more effective and personalized therapeutic options.

9. The Role of MiR-9 in Drug Resistance

One of the most important areas of cancer research involves the issue of drug sensitivity and or drug response in the clinical setting. In many tumor settings, patients can either be initially responsive to certain therapies or completely refractory. Moreover, in certain contexts patients are living longer post cancer diagnosis and therefore are facing the challenges of acquired drug resistance resulting in limited options for extended treatments within these patients. As an example, in lung cancer, the leading cause of cancer-related deaths worldwide, a majority of patients that respond to cisplatin and radiation therapy eventually relapse. Clinicians have struggled to develop newer therapies for these patients. Recently, the aberrant expression of

certain miRNAs are now known to be associated with therapeutic resistance [275-279]. In the case of miR-9, A549 lung cancer cells expressing miR-9 resulted in radiosensitization. Furthermore, miR-9 also prevents A549 cells from migrating and proliferating efficiently. These studies have not fully interpreted the miR-9 isoforms involved, as well as the exact genetic mRNA targets that contribute to these phenotypes, therefore more work is required to elucidate this chemoresistant biology.

In the breast cancer setting, the role of miR-9 is more evident, yet more confounding. As mentioned above miR-9 can regulate both hormone responsiveness and receptor kinase pathways important in maintaining tumorigenesis. Despite these disparate responses, many still view miR-9 as an oncogenic miRNA that supports tumorigenesis. In support of this, in MCF-7 cells the exosomal transfer of miR-9-5p, as well as other miRNAs, enhanced the resistance of MCF-7 cells to tamoxifen [207,277]. The ability for miR-9-5p to target and downregulate ADIPOQ is quite relevant given adiponectin signaling is known to modulate AMPK and P38 MAPK signaling pathways that are known to support cytoprotection, glucose uptake, and fatty acid oxidation in the absence of hormone signaling. This indicates that specifically expressing a decoy miR-9-5p isoform in patients with acquired tamoxifen resistance may extend curative latency within these patients.

In other cancer types, the cellular mRNA repertoire completely changes depending upon the evolutionary bottleneck, caused by the chemotherapeutic action on the tumorigenic cell type. For instance in CLL, the use of adriamycin (ADR) results in initial cytotoxicity, yet these tumor cells typically express multi-drug transporters such as ABCB1 so as to prevent the ADR compound from operating on the tumor DNA [278]. Therefore an RNA therapy that targets ABCB1 may be a logical approach in re-sensitizing these cells to ADR. In glioblastoma, the agent of choice in the clinic for over thirty years is still TMZ [280], which results in an initial and robust apoptosis of glioma cells. Over time however, most gliomas become refractory to any chemotherapeutic agent utilized. Given the role of miR-9 in gliomagenesis, researchers should further assess if miR-9 alters the response of these tumors to TMZ. Interestingly, in clinical settings, the re-expression of miR-9 was sufficient to reverse cancer cell chemoresistance. This is true in the case of re-sensitizing ADR-resistant K562 CML cells to ADR, as well as increasing the apoptotic rate of glioma cells when simultaneously given TMZ, however, the target mRNA genes involved in this re-sensitization process are still too numerous to fully understand the genetic driver(s) of chemoresistance.

Many of these chemosensitization studies are still in the preliminary stages of development, and therefore few researchers have gone back to understand how various miR-9 isoforms regulate acquired drug resistance in their stable cancer cell models. In one study, researchers found that both miR-9-5p and miR-9-3p can actually target DNA topoisomerase IIα (TOP2α) [281]. TOP2α is a decatenating enzyme that generates double stranded DNA breaks during DNA repair. Tumor cells tend to down regulate this gene, allowing for genomic mutations to occur, facilitating tumorigenesis. Interestingly, both miR-9 variants can bind and target TOP2a in leukemia, indicating the miR-9 loci operates as a *bona-fide* oncogene. More importantly, miR-9-5p and miR-9-3p become elevated when K562 cells are treated with etoposide. Furthermore, the transfection of synthetic miR-9-3p or miR-9-5p inhibitors in etoposide-resistant K562 cells resulted in enhanced etoposide-induced DNA damage and increased TOP2α protein levels. These studies emphasize the importance of the cellular context of a particular cancer, and the cell-specific miRNA and mRNA repertoire present after acquired drug resistance. When these intricacies are fully understood, effective therapeutic strategies can be employed that utilize synthetic miRNA oligonucleotides so as to effectively alter the cellular mRNA repertoire, such that drug resistance cells can become re-sentitized to chemotherapeutic compounds that are considered safe, and effective as determined by clinical standards.

10. Conclusion

The story of miR-9 showcases the inherent duality of miRNA functionality, acting as both a tumor suppressor and an oncogene across various cancers. The role of miR-9 is highly context-dependent, and is shaped by the specific genetic, epigenetic, and molecular environments of these different tumor types. As a tumor suppressor, miR-9 is downregulated through mechanisms such as hypermethylation, resulting in the ability of oncogenic pathways controlled by miR-9 to be activated, resulting in cancer development. Furthermore, miR-9 overexpression can at times promote tumorigenesis by suppressing key tumor suppressors in malignancies such as MLL-rearranged acute myeloid leukemia and glioma. Interestingly, miR-9 can also operate as an oncogene in some cancers through targeting genes that support acquired drug resistance. The dual role of miR-9 is in part explained by the differential cellular mRNAs that predominate in the cell at any given point in time, but also by the two major miR-9 variants miR-9-5p and miR-9-3p being differentially co-expressed in these cells, each with vastly different cellular mRNA targets. However, additional miR-9 variants exist, and highlights the molecular complexity of miR-9 and the further necessity to elucidate the mechanism of action with respect to miR-9 as a therapeutic agent. Importantly, development of miR-9-based therapies–whether through restoring expression in cancers where miR-9 functions as a tumor suppressor, or inhibiting function in cancers where miR-9 behaves as an oncogene–offers a promising option for precision oncology. While significant advancements have been made in understanding the role of miR-9, further research is needed to overcome the delivery, stability, and specificity challenges associated with miRNA therapeutics. By enhancing our knowledge of miR-9 biology, researchers can use miR-9 as a diagnostic marker and therapeutic target, leading to novel cancer treatments tailored to the molecular profile of patients.

Ethical Approval

Not applicable in this study.

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Consent to Publish

Authors give their consent to publish this manuscript upon acceptance.

Conflict of Interest

Authors declare no conflict of interest.

Author Contributions

M.I., M.A., A.A., E.S., and B.A. performed the literature research and co-wrote the manuscript. G.S., J.I., N.R., and H.D. provided conceptual editing support, and developed the figures and tables for the manuscript. L.G., Z.F., A.P., Y.C., and B.A. reviewed and editorially revised the manuscript. B.A. reviewed and provided advice on the content of this manuscript.

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