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Review

A Systematic Review of miR-29 in Cancer

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MicroRNAs (miRNA) are small non-coding RNAs (∼22 nt in length) that are known as potent master regulators of eukaryotic gene expression. miRNAs have been shown to play a critical role in cancer pathogenesis, and the misregulation of miRNAs is a well-known feature of cancer. In recent years, miR-29 has emerged as a critical miRNA in various cancers, and it has been shown to regulate multiple oncogenic processes, including epigenetics, proteostasis, metabolism, proliferation, apoptosis, metastasis, fibrosis, angiogenesis, and immunomodulation. Although miR-29 has been thoroughly documented as a tumor suppressor in the majority of studies, some controversy remains with conflicting reports of miR-29 as an oncogene. In this review, we provide a systematic overview of miR-29's functional role in various mechanisms of cancer and introspection on the contradictory roles of miR-29.

MicroRNAs (miRNAs) are small non-coding RNAs, approximately 22 nt in length, that are known as powerful regulators of gene expression in eukaryotes. Since the first miRNA, lin-4, was reported in 1993,[1] the field of miRNA biology has exploded in the past quarter century, as revelations were made that these minute RNAs had colossal implications in a multitude of physiological processes and diseases. In the context of cancer pathogenesis, miR-15a/miR-16 was the first miRNA cluster found to be aberrantly regulated, as their encoding genomic region was found to be deleted in chronic lymphocytic leukemia (CLL).[2] Since then, dysregulated miRNA signatures have become a well-established feature of various cancers. Among dozens of miRNAs that have been reported to be abnormally expressed in cancer, miR-29 has been recognized as one of the critical miRNAs that play a role in cancer pathogenesis.

miR-29 has been shown to have an important role in a multitude of pathophysiological processes, ranging from cardiovascular[3] to retinal functions[4] and even Alzheimer disease (AD).[5] An increasing number of *in vivo* and *in vitro* studies have demonstrated miR-29 to exhibit strong anti-fibrotic activity by negative regulation of mRNAs encoding extracellular matrix (ECM) proteins, such as collagen type I, alpha 1 and 2 (COL1A1, COL1A2), collagen type III alpha 1 (COL3A1), elastin (ELN), and fibrillin 1 (FBN1), which play essential roles in matrix deposition, epithelial-mesenchymal transition (EMT), and the progression of fibrosis.[6,7] Consistently, miR-29 is significantly downregulated in human fibrotic disorders of multiple organs. For example, in cardiac, pulmonary, hepatic, and renal fibroses, miR-29 is found to be downregulated by transforming growth factor β (TGF-β)-SMAD signaling, which in turn results in enhanced expression of the collagen proteins, promoting pathogenesis of the disease.[7–12] In hepatic stellate cells (HSCs), key contributors of collagen production and fibrogenic reactions in liver, miR-29 overexpression markedly inhibits the increased expression of HSC-activating genes α-SMA, DDR2, FN1, ITGB1, and platelet-derived growth factor receptor (PDGFR)-β *in vitro*.[13] Taken together, deregulation of miR-29 is associated with conditions such as myocardial fibrosis, cardiac hypertrophy, congestive heart failure, chronic hepatic injury, hepatitis C virus (HCV) infection and inflammation, hypertensive and diabetic nephropathies, and chronic kidney diseases.[3,12,14–18]

In addition to fibrogenic disorders, miR-29 is reported to negatively regulate insulin signaling via the inhibition of insulin receptor substrate 1, phosphoinositide 3 kinase, and hexokinase 2, thereby playing a pivotal role in glucose and fatty acid metabolism and type 2 diabetes.[19,20] Furthermore, recent studies have delineated the role of miR-29 in neurodegenerative diseases such as AD.[21] In AD, patients exhibit high levels of beta-secretase 1 (BACE1), an enzyme responsible for β-amyloid peptide (Aβ) formation from amyloid precursor protein gene (*APP*). Loss of miR-29 results in an associated increase in BACE1 expression and Aβ levels, promoting AD pathogenesis.[5,21,22]

In addition to the aforementioned diseases, a large body of literature has demonstrated the significant role of miR-29 in various cancers. The majority of these studies have reported that miR-29 functions as a potent tumor suppressor gene, yet a few other reports have also found oncogenic function of miR-29. To comprehensively summarize the role of miR-29 in cancer, we have curated >150 primary research articles pertaining to miR-29 spanning 13 different cancer types. The search strategy was directed toward English language articles obtained through the PubMed electronic database to identify peer-reviewed articles published and available to date. Here we offer a systematic review synthesizing the current findings of miR-29 and its roles in various mechanisms of cancer.

miRNA Biology and the miR-29 Family

miRNAs are normally transcribed by RNA polymerase II and are commonly embedded in the introns and exons of both coding and

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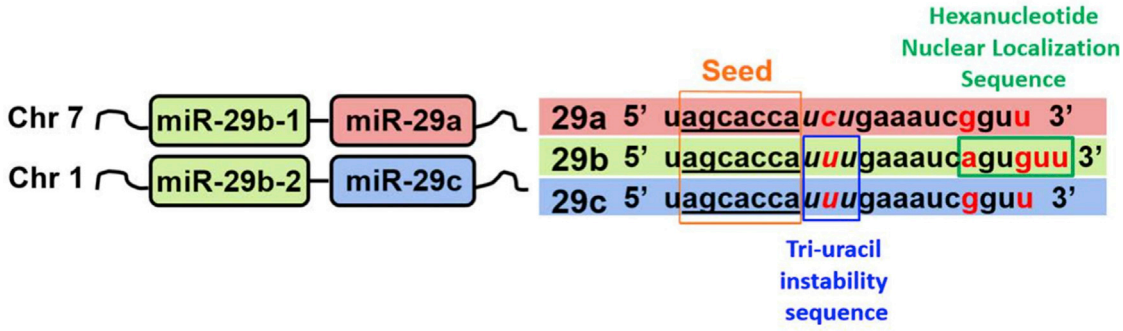

Figure 1. Schematic Representation of the miR-29 Family Members: miR-29a, -29b, and -29c

miR-29 family members have identical seed sequences (orange box and underlined) along with similar mature miRNA sequences. However, notable differences in nucleotides are indicated in red. Tri-uracil nucleotides at positions 9–11 nt (blue box) present in miR-29b and -29c contribute to instability and shorter half-life, and the hexanucleotide sequence at positions 18–23 nt (green box) is unique to miR-29b, leading to nuclear localization.

non-coding genes.[23] Within these transcripts, they form a characteristic hairpin structure and are referred to as primary miRNAs (pri-miRNAs).[23] Subsequently, pri-miRNAs are processed by ribonuclease enzyme Drosha in conjunction with a co-factor (DiGeorge syndrome critical region in gene 8 [DGCR8] in humans or Pasha in *Drosophila melanogaster* and *Caenorhabditis elegans*), forming the microprocessor complex, which cleaves at the base of the hairpin.[24,25] This liberated 60- to 70-nt hairpin structure is referred to as precursor miRNA (pre-miRNA), which is exported out of the nucleus by the RanGTP-dependent nuclear transport receptor exportin 5[26,27] and further processed in the cytoplasm by an endonuclease RNase III enzyme, Dicer, to yield an ∼22-nt long double-stranded mature miRNA composed of a 5 prime (−5p) and a 3 prime (−3p) strand.[28,29] Finally, one of these strands of mature miRNA (passenger strand) is degraded, while the other strand (guide strand or miRNA) is loaded onto the Argonaut proteins (AGOs) to form the RNA-induced silencing complex known as RISC.[30,31] In the case of miR-29, the miR-29-3p arm represents the most abundant and functionally relevant arm of all three family members.[32,33] Following its association within RISC, miRNAs function through a 6-8-mer sequence, known as a seed sequence, that complementarily binds to the 3′ UTR of mRNA transcripts through canonical Watson-Crick base pairing to regulate target expression.[23,27] miRNA biogenesis is a highly complex machinery, and we have provided a very simplified overview of the entire process. A more detailed review of miRNA biogenesis is available for further reading.[25,27,29,31]

Several miRNAs have common seed sequences and have been known to largely regulate a similar repertoire of target transcripts. Accordingly, evolutionarily conserved miRNAs sharing analogous seed sequences have been organized into 87 distinct miRNA families.[34] One of these miRNA families, miR-29, consists of three members, miR-29a, miR-29b, and miR-29c. miR-29a and -29b-1 are encoded on human chromosome 7q32.3, known as the miR-29a/b-1 cluster, and miR-29b-2 and -29c are found on chromosome 1q32.2, designated as the miR-29c/b-2 cluster. While all three family members share a common 7-nt seed sequence, there are unique sequence-functional features of each miR-29 family member that have been reported (Figure 1).

One of the notable differences among miR-29 family members resides within a 6-nt segment that is unique to miR-29b (Figure 1). This hexanucleotide sequence present in nucleotide positions 18–23 has been functionally shown to lead to miR-29b nuclear localization.[35] In fact, the simple addition of the miR-29b nuclear localization sequence (NLS) to the 3′ end of small interfering RNAs (siRNAs) was found to be sufficient enough to cause nuclear localization.[35] Consistently, another report, evaluating the therapeutic efficacy of miR-29b in targeting a subunit of the proteasome, found that the deletion of the hexanucleotide NLS led to cytoplasmic enrichment of miR-29b and further enhanced the downregulation of its target.[36] Although miR-29b's function within the nucleus has yet to be fully elucidated, this initial work has opened the gates to the discovery of various other miRNAs that similarly localize to the nucleus and elicit non-canonical gene regulation beyond binding to the 3′ UTR of mRNA transcripts in the cytoplasm.[37,38] The study of miRNA nuclear function is still active and evolving. A number of reviews are available on this subject matter,[39,40] but, for simplicity, we will focus on the canonical-cytoplasmic function of miR-29 for the remainder of this review.

In addition, miR-29a has a distinct cytosine residue at position 10 (Figure 1). This difference is of particular importance, as uracil residues located in nucleotide positions 9–11 of miRNAs have been found to lead to rapid decay or turnover.[41] This tri-uracil sequence is found in both miR-29b and miR-29c, whereas the cytosine residue at nucleotide position 10 of miR-29a lends to its greater stability.[41] This is consistent with pulse-chase experiments of synthetic miR-29a and -29b in HeLa cells demonstrating a longer half-life of miR-29a compared to -29b,[36] in addition to numerous findings demonstrating that miR-29a is the most abundantly expressed family member.[42–45]

In Vivo Function of miR-29

A single miRNA family has been known to have, on average, >400 target transcripts that have matching, evolutionarily conserved

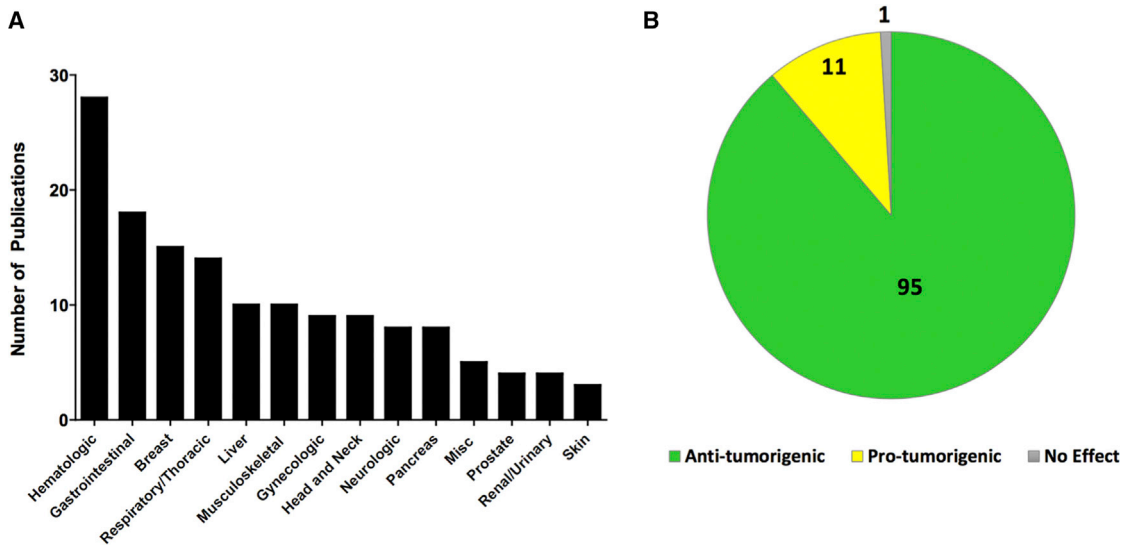

Figure 2. miR-29 Studies in Cancer Pathogenesis

A systematic curation of English language, primary research articles related to miR-29 in the context of cancer were comprehensively surveyed using the PubMed electronic database in order to gain a better insight into the role of miR-29 in various cancers. (A) Bar graph indicating the number of existing publications based on cancer type, with Miscellaneous (Misc) indicating studies that investigate miR-29 in a broader, multi-cancer context. (B) Pie chart with the numbers of publications demonstrating miR-29 as a tumor suppressor (green), oncogenic (yellow), and having no effect (gray).

3′ UTR-binding sites.[46] Furthermore, >60% of translated genes in the human genome possess at least one miRNA-binding site. Therefore, it is not difficult to imagine that dysregulation of even a single miRNA can profoundly impact normal physiological processes and lead to disease.

In fact, a number of *in vivo* studies in murine models have demonstrated the essential role of miR-29 in development and general physiology. Whole-body miR-29 knockout (KO) mice have a wide range of developmental defects, such as premature thymic involution,[47] muscle wasting,[48] growth retardation, and shorter lifespan.[49] In addition, liver-specific miR-29 KO mice had a robust increase in hepatic fibrosis and carcinogenesis,[16] implicating its physiological relevance.

A recent report profiled miR-29a and miR-29c function in the pancreata of whole-body KO mouse models in the context of glucose regulation and diabetes.[44] miR-29a KO mice had a defect in insulin secretion, but, interestingly, miR-29c KO mice did not.[44] Similarly, miR-29a KO in an insulitis transgenic model (insHEL) resulted in diabetes, whereas wild-type (WT) mice did not.[44] Taken together, miR-29a plays a vital role in proper pancreatic function and elicits a protective effect against diabetes in the context of insulitis.[44] miR-29 clearly has a critical role in various organs *in vivo* to help maintain homeostasis and normal function, and its loss leads to developmental problems and disease. Thus, it is not surprising that the misregulation of miR-29 is reported in a variety of different cancer types (Figure 2A) and that it predominantly functions as a tumor suppressor (Figure 2B).

Epigenetics

The genome is more than just the simple sequence of its nucleotides. The term epigenetics was originally coined in 1942,[50] where the regulatory mechanism of gene expression was considered as a transcending process to explain phenotype. Epigenetics is the study of higher-order regulatory mechanisms influencing the expression of genes without altering the underlying sequence. Since the original conception of the term in 1942, the field of epigenetics has expanded in the past several decades to include a multitude of molecular mechanisms (e.g., histone modifications, DNA methylation, miRNAs, and long non-coding RNAs [lncRNAs]).

DNA methylation is a well-studied mechanism of epigenetic regulation, and it has been shown to be aberrantly regulated in cancer. Methylated DNA effectively silences genes through preventing the association of activator proteins and recruiting methyl-CpG-binding proteins (MBD) to deacetylate neighboring histones. DNA methylation of CpG islands is catalyzed by a class of proteins known as DNA methyltransferases (DNMTs). Furthermore, DNMT family members (DNMT1, DNMT3A, and DNMT3B) have been shown to be elevated in a number of different cancers.[51–54]

As one of the earliest implications of miR-29's role in epigenetic regulation, all three miR-29 family members were found to act as tumor suppressors in lung cancer through the direct targeting of a 3′ UTR-binding site on *DNMT3A* and *DNMT3B* transcripts.[55] This was of particular interest as DNMT3 was shown to be increased in lung cancer and promote tumor growth by silencing various tumor suppressor genes.[51] Further *in vivo* studies demonstrated miR-29's ability

to effectively repress tumor growth in a xenograft mouse model.[55] Various other reports followed, corroborating the tumor-suppressive role of miR-29 through targeting DNMT3s in other cancer contexts (Table S1).

In addition to DNA methylation, the post-translational modification of histones is another major epigenetic regulatory mechanism. Due to the negatively charged phosphate backbone, DNA naturally associates with the positively charged lysine and arginine residues of histone proteins, effectively packaging the DNA into units called nucleosomes.[56] In the packed heterochromatic state, the DNA associated with histones is effectively inaccessible, and promoter regions or DNA elements within these regions are turned off. The acetylation of lysine residues on the N-terminal tails of histones can neutralize the positive charge of the side chain group, thus loosening its association with DNA. This leaves the DNA more open to transcriptional regulators. The reaction of acetylating lysine residues of histones is facilitated by a class of enzymes called histone acetyltransferases (HATs). Conversely, the removal of acetyl groups is carried out by histone deacetylases (HDACs). HDACs have been shown to promote oncogenic processes and are considered therapeutic targets in a multitude of cancers.[57,58] In fact, several clinical trials are ongoing in an attempt to implement HDAC inhibitors (HDACi) in combination with other therapeutic agents (ClinicalTrials.gov: NCT01543074 and NCT01486277).

In a study involving multiple myeloma, synthetic miR-29b mimics were shown to effectively inhibit class II *HDAC4* expression,[59] and the 3′ UTR miR-29-binding site was validated through luciferase reporter assay. Furthermore, miR-29b expression was inversely correlated with *HDAC4* expression in various cancer cell lines as well as in patient samples.[59] The study went on to demonstrate that miR-29b overexpression caused increased acetylation of histone H4, increased apoptosis, and reduced migratory potential of myeloma cells. Interestingly, HDAC inhibition also led to the upregulation of miR-29b, indicating a feedforward mechanism. Finally, the administration of miR-29b mimics in combination with a pan-HDAC inhibitor, Vorinostat, led to a significant increase in survival and decreased tumor volume *in vivo*.[59]

In addition to miR-29 targeting *DNMT3* and *HDAC4*, Thymine DNA glycosylase (*TDG*) and ten-eleven translocation 1 (*TET1*) have been indicated as direct targets of miR-29. TDG and TET1 are known to facilitate active demethylation of DNA, where TET1 initiates DNA demethylation, through hydroxylating methylated cytosine,[60,61] and TDG subsequently excises the modified methyl group, effectively demethylating DNA.[62]

One of the first reports of miR-29 targeting *TET1* and *TDG* was in the human lung adenocarcinoma cell line A549.[63] The miR-29-binding sites within the 3′ UTR of *TET1* and *TDG* were validated via luciferase reporter assay.[63] Consistently, *TET1* and *TDG* targeting by miR-29, in the context of various cancers through bioinformatics analysis of data, was identified with The Cancer Genome Atlas (TCGA) data.[64] Using a statistical method to derive recurrence scores between miRNAs and mRNAs in complement to target prediction, a database of high-confidence miRNA pathway networks for multiple cancer types was created (http://cancerminer.org). As a proof of concept for their analysis, the authors described a potential regulatory role of miR-29 in the DNA demethylation pathway through targeting *TET1* as well as *TDG*.[64]

Interestingly, TET1 has been shown to act as a potent tumor suppressor in various oncogenic contexts.[65,66] A study in breast cancer cell lines revealed miR-29b overexpression led to increased proliferation, colony formation, and migration through inhibiting TET1 to regulate ZEB2.[67] In contrast, thorough analysis of TCGA datasets across a number of cancer types revealed that miR-29a is generally downregulated, and *TET1* and *TDG* are typically upregulated.[64] These seemingly contradictory results led to the idea that miR-29's role in epigenetic regulation as a tumor suppressor versus oncogene may be more subtle and context dependent. However, it is clear from the given studies that *TET1* and *TDG* are bona fide targets of miR-29. More recent work in stem cell differentiation has shown consistent results of miR-29 effectively targeting *TET1* in embryonic stem cell differentiation.[68,69] Further work is needed to better understand these paradoxical findings.

Proteostasis

As the cell is challenged with various environmental stresses, maintaining a balance of protein biogenesis and degradation, called proteostasis, is a vital process in preserving cellular homeostasis. The loss of this balance can lead to dysfunction and cell death. Cancerous cells undergo a constant barrage of environmental stresses, including hypoxia and nutrient deprivation, while meeting the demands of oncogenic growth. Therefore, proteostasis is critically important for the survival of transformed cells, and it has been proposed as an appealing therapeutic target. Therapeutic targeting of proteostasis in the context of cancer have been covered in several reviews.[70–73] The proteasome and the process of macroautophagy are essential for protein clearance and turnover, and, in recent years, miR-29 has emerged as a novel regulator of proteostasis by targeting several genes that are pertinent to proteasome and autophagy pathways.

The proteasome is a protein complex that hydrolyzes peptide bonds of polyubiquitinated proteins.[72] The ubiquitin proteasome pathway (UPP) is vital for regulating protein half-life and degrading damaged or misfolded proteins.[72] In complement to the proteasome, macroautophagy, herein referred to as autophagy, is a key cellular process that degrades large macromolecules for the maintenance of cellular homeostasis and survival under stress conditions.[74] Autophagy is the process in which cytosolic components are enveloped by double-membrane vesicles, called autophagosomes, and trafficked to the lysosome for degradation and recycling.[75] Subsequently, the hydrolases of the lysosomal compartments degrade cytoplasmic cargo and release the degraded components into the cytosol for reuse.[75] Recent studies document that the upregulation of autophagy can serve as a survival mechanism in various malignancies.[76–87] As cancer

frequently relies upon the UPP and autophagy, the development of proteasome inhibitors and small molecule inhibitors of autophagy for cancer therapy is an active area of research.

The first indication of miR-29's role in proteostasis was reported in the context of chemosensitizing myeloma to bortezomib, a therapeutic proteasome inhibitor.[36] Initially, there was an aim to screen several thousands of miRNAs differentially expressed in bortezomib-resistant cells, and miR-29a, 29b-1, miR-29b-2, and miR-29c were all identified as significantly downregulated miRNAs. Upon the inhibition of miR-29b and miR-29c using antagomirs, the viability of bortezomib-treated myeloma cells was found to be increased. miRNA target prediction algorithms revealed a potential target involved in proteasome activation called *PSME4* as a direct target of miR-29b. Finally, utilizing transgenic xenograft murine models, miR-29b replacement was shown to decrease tumor volume and increase overall survival. Although further studies have not investigated miR-29 suppression of PSME4 in the context of cancer, another recent report found similar results in the context of cardiovascular disease, where miR-29b overexpression led to a PSME4 decrease and increased oxidative stress.[88]

In addition to the proteasome, miR-29 has been shown to play a significant role in regulating autophagy. Initial studies in bovine cells infected with virus revealed endogenous bovine miR-29 had an anti-viral protective effect of the host cell by upregulating miR-29b, which led to the targeting of *ATG14* and *ATG9A*. Bovine diarrhea virus (BVDV) utilized host cell autophagy machinery as a means of promoting viral replication. miR-29b-mediated inhibition of autophagy led to a decrease in BVDV replication.[89]

Shortly thereafter, miR-29a was shown to have a similar effect inhibiting autophagy in pancreatic cancer cells. Earlier work demonstrated that pancreatic cancer cells heavily upregulated autophagy for survival and that this upregulation also conferred chemoresistance against a nucleoside analog, gemcitabine.[87] miR-29a was significantly downregulated in pancreatic cancer cells, and miR-29a overexpression led to increased gemcitabine sensitization in chemoresistant cell lines. Subsequently, miR-29 overexpression led to a late-stage blockage of autophagy, where co-localization of GFP-labeled autophagosomes and lysosomal markers indicated a blockage in autophagosome-lysosome fusion. By utilizing miRNA predictive algorithms, two predicted autophagy-related miR-29 targets were identified, *TFEB* and *ATG9A*. TFEB and ATG9A are known to be vital for lysosomal function and autophagosome trafficking, respectively, and they are indispensable for autophagy. Indeed, miR-29 overexpression led to a drastic decrease in expression of both targets. Parallel knockdown of TFEB and ATG9A phenocopied the late-stage blockage of autophagy similar to miR-29 overexpression, but, interestingly, only the knockdown of ATG9A demonstrated a reduction in autophagosome-lysosome fusion.

While initial findings of miR-29b targeting bovine *ATG9A* were originally reported, we found consistent regulation in human carcinoma. Furthermore, in a study involving the silencing of HDAC4 by miR-29b in multiple myeloma, miR-29 overexpression led to a decrease in TFEB.[59] Although postulated that this was an indirect effect of repression through the knockdown of HDAC4, it may be possible they were, in fact, observing a direct targeting of TFEB by miR-29b. However, in subsequent studies, overexpression of miR-29a in myeloma cells had no impact on autophagy flux.[59] These results may indicate that miR-29-mediated autophagy inhibition may be context dependent or differ between miR-29a and -29b family members. Further investigation is warranted to determine if miR-29 has an impact on proteasome function in pancreatic cancer.

Oncogenic Metabolism

miR-29 has been implicated in regulating metabolism in numerous tissue types, including β cells, skeletal muscle, and adipocytes,[20,90,91] and it has been shown to regulate branch-chain amino acid synthesis and insulin secretion.[91,92] Therefore, it is not surprising that miR-29 is also found to have an impact on cancer metabolism. The area of cancer metabolism has been well studied over the past several decades, and many alterations in metabolic processes, including glycolysis, oxidative phosphorylation, and mitochondrial function, have been discovered as a compensatory mechanism of cancer cells to meet the energy demands of oncogenesis.

One of the first studies elucidating the role of miR-29 in cancer metabolism found the downregulation of miR-29b in ovarian cancer, and its restoration led to an inhibition of glycolysis and glucose metabolism in cancer cells by directly targeting *AKT2* and *AKT3*.[93] AKT, also known as protein kinase B, is a key hub within the PI3K/AKT/mTOR-signaling pathway. Furthermore, AKT is well known to be activated in tumors and regulates glucose metabolism to promote cancer growth.[94,95] The 3′ UTR miR-29-binding sites of *AKT2/3* were validated, and miR-29b overexpression led to decreased tumor formation *in vivo*.[93] Although outside of the metabolism context, various other groups have demonstrated a similar tumor-suppressive function of miR-29 by targeting *AKT* (Table S1), thus reassuring that *AKT* is a bona fide target of miR-29.

In addition to facilitating a tumor-suppressive function through regulating glucose metabolism, miR-29 has also been shown to be concertedly regulated in the context of lipid metabolism, and it functions as a tumor suppressor in glioblastoma multiforme (GBM) through an autoregulatory loop involving Sterol regulatory element-binding protein 1 (SREBP-1).[96] SREBP-1 is a transcription factor that concertedly regulates genes involved in sterol biosynthesis and glucose and lipid metabolism,[97] and it has been known to promote tumorigenesis.[98] SREBP-1 was found to suppress miR-29 expression at both loci (miR-29a/b-1 and miR-29c/b-2), and deletion of sterol regulatory element (SRE)-binding sites in the promoters of miR-29 loci derepressed miR-29 expression.[96] Interestingly, *SREBP1* itself was shown to be a direct target transcript of miR-29, and, ultimately, miR-29 forced expression in GBM led to a significantly increased survival and decreased tumor growth *in vivo*.

Finally, miR-29 has also been implicated to directly target *ATGP5G1* and *ATPIF1*, two vital subunits of ATP synthase.[99] The overexpression of miR-29a/b-1 caused tamoxifen sensitization in tamoxifen-resistant cells.[99] Gene ontology analysis of the miR-29a transcriptome in ovarian cancer cells revealed various differentially expressed genes (DEGs) related to oxidative phosphorylation and ATP metabolism.[99] Further investigations revealed miR-29a overexpression led to a decreased oxygen consumption rate, mediated by the direct inhibition of ATP5G1 and ATPIF1 expression.[99] Although these findings necessitate further *in vivo* verification, they convey additional evidence of miR-29's tumor-suppressive role in regulating cancer metabolism.

Proliferation

Uncontrolled proliferation is a hallmark of cancer cells, and several cell cycle regulators are well known to be dysregulated in tumors. Understanding the molecular details of cell cycle regulation and checkpoint abnormalities in cancer have been under intense investigation for the past several years. The targeting of cell cycle control mechanisms has risen as a promising therapeutic strategy.[100]

The main regulatory proteins that play key roles in controlling cell-cycle progression include cyclins, cyclin-dependent kinases (CDKs), and various cyclin substrates.[101] CDK6 plays an important role in the transition of cycling cells into S phase. Studies have reported that cyclin-CDK6 complexes induce cancer cell transition from G1 to S phase by phosphorylation of retinoblastoma (Rb), while CDK6 inhibition blocks the cell cycle and suppresses tumor growth.[102,103] In fact, several CDK6 inhibitors have been developed to target proliferating cancer cells.[104] *CDK6* has been shown to be a direct target of miR-29 in several malignancies, including mantle cell lymphoma,[105] acute myeloid leukemia (AML),[106] and cervical cancer.[107] For example, *CDK6* was shown to be directly targeted by miR-29 in melanoma cell lines. In various melanoma cell lines, miR-29a/b expression inversely correlated to cancer cell proliferation. Previous work had demonstrated an anti-proliferative effect of interferon (IFN)-γ in many cancers.[108] Accordingly, IFN-γ as well as miR-29 exhibit anti-proliferative activities in melanoma cells involving downregulation of *CDK6*.[109] Consistently, miR-29 was shown to be involved in Burkitt lymphoma pathogenesis by altering the expression of target genes, including *CDK6*, *DNMT3B*, *TCL1*, and *MCL1*, which are involved in cell cycle control, DNA methylation, and apoptosis inhibition, respectively.[110] Moreover, miR-29 has also been observed to enrich the E7 protein-dependent cell cycle pathway by targeting CDK6, resulting in decreased proliferation of cervical cancer cells.[111] Furthermore, cell cycle analysis revealed that miR-29 overexpression caused a significant accumulation of cervical cancer cells at the G1 phase and decreased the number of cells in the S and G2/M phases.[112] In schwannoma, miR-29a has been shown to downregulate CDK6 expression and disrupt cell cycle progression by the deactivation of JNK and p38MAPK/ERK pathways. Overexpression of all three members of the miR-29 family in Schwann cells inhibited cell viability, migration, and invasion *in vitro*.[113]

Interestingly, a recent study identified a novel oncogenic circular RNA, circRNA_100290, which contained two miR-29-binding sites that acted as a sponge to sequester miR-29 family members in oral squamous cell carcinoma (OSCC).[114] The inhibition of miR-29b led to the upregulation of CDK6 expression. Knockdown of circRNA_100290 was found to induce G1/S arrest and inhibit *in vitro* and *in vivo* OSCC cell proliferation and reduce CDK6 expression.

Another miR-29 target, cell division cycle 42 (*CDC42*), is a well-known member of the Ras homolog (Rho) family, and it regulates crucial cellular processes, including cell cycle and cell cytoskeleton organization.[115,116] miR-29a was shown to exert a tumor suppressor role in breast cancer cells by arresting the cell cycle at the G0/G1 phase through the negative regulation of *CDC42* expression.[117] In corroboration with this study, miR-29 was found to be downregulated in clear cell renal cell carcinoma, and restoration of all three miR-29 family members inhibited cell proliferation along with migration-invasion.[117]

As covered in the Oncogenic Metabolism section, the AKT kinase family is well known for its role in promoting tumorigenesis. One mechanism by which AKT functions as an oncogene is through promoting cell proliferation by accelerating G2-M phase transition.[118] miR-29 has been shown to inhibit myoblast proliferation by targeting *AKT3* as well as another proliferation-associated gene, *p85α*. siRNA-mediated *AKT3* knockdown in the C2C12 myoblast cell line was shown to phenocopy miR-29 overexpression, leading to cell-cycle arrest in the G0/G1 stage and a significant decrease in proliferation rate.[119] In addition to *AKT3*, *AKT2* and *CCND2* have also been shown to be direct targets of miR-29. AKT2 functions as the hub in the PI3K/AKT-signaling pathway,[120–122] and CCND2 is a member of the cyclins, promoting the G1-to-S phase transition through regulating the phosphorylation of Rb.[123,124] The role of miR-29 in significantly inhibiting AML cell proliferation and promotion of apoptosis was attributed to the decrease of these two key signaling molecules.[125] Furthermore, *in vivo* analysis demonstrated that the reintroduction of each miR-29 member could partially correct abnormal cell proliferation and apoptosis repression and mediate myeloid differentiation arrest in AML.[125]

In addition to *CCND2*, miR-29 also targets *E2F7*, as demonstrated in Rhabdomyosarcoma (RMS). E2F7 is essential for cell survival and embryonic development in mice. Furthermore, ectopic expression of *E2F7* has been shown to block cell cycle transition, resulting in G1 arrest.[126,127] *CCND2* and *E2F7* were upregulated in both RMS types, and miR-29 expression was shown to be inversely correlated to these cell cycle genes, suggesting that miR-29 may function as a tumor suppressor in RMS by targeting *CCND2* and *E2F7*. Ultimately, overexpression of miR-29 downregulated the expression of these cell cycle genes and induced partial G1 arrest, leading to decreased cell proliferation.[128]

Finally, miR-29 also targets *FOXM1*. FOXM1 is a conservative transcription factor that can mediate cell growth, proliferation, and apoptosis through regulating gene expression.[129] A study demonstrated

that the overexpression of miR-29 inhibited human leukemia K562 cell growth and proliferation and promoted apoptosis by downregulating FOXM1 expression level.

Apoptosis

The apoptotic pathway is an important pathway in all stages of tumor development and metastasis. Apoptosis is a naturally acquired process that typically plays an important role in the development and life of multicellular organisms, by removal of damaged, aged, or autoimmune cells through a controlled cell death mechanism.[130] Apoptosis involves a fine-tuned regulatory mechanism, as it is categorized as a type I form of programmed cell death; therefore, the mechanism of apoptosis is complex and involves numerous signaling pathways. Critical changes may occur at any point along these pathways, leading to tumorigenesis, metastasis, and resistance to anticancer drugs.[131,132] A large body of experimental evidence documents that miRNAs function as important regulators of cancer cell death. In an effort to re-establish miRNAs function in apoptotic pathways, many strategies have been designed to either block the expression of oncomiRs or to boost the expression of tumor suppressor miRNAs.[133]

miR-29a and -29b have been shown to regulate critical anti-apoptotic genes such as *MCL1*. MCL1, part of the BCL family, is an anti-apoptotic protein that promotes cancer cell survival and proliferation, and it is frequently overexpressed in cancer, notably in AML.[134–137] miR-29b has been shown to be deregulated in primary AML blasts, and transcriptome analysis after miR-29b overexpression in leukemia cells revealed a tumor suppressor function, in which miR-29 induced apoptosis through directly targeting *MCL1*, as indicated by Annexin V/propidium iodide (PI) assay and increased caspase activity.[138] These results of miR-29b targeting *MCL1* to elicit a tumor suppressor effect were consistent *in vivo*.[138] In addition, an inverse relationship of miR-29 and MCL-1 expression in the transformation of myelodysplastic syndromes (MDS) to overt leukemia (OL) was observed.[138] Furthermore, miR-29a has been shown to have anti-invasive and anti-proliferative effects on lung cancer cells *in vitro*, which can be explained in part by the ability of the miR-29 family to downregulate Mcl-1 activity and target RAN, a member of the RAS oncogene family.[139]

miR-29 also regulates environmental signals like growth factors that have an extrinsic impact on cell cycle control. It was found that the knockdown of miR-29 inhibited proliferation and induced apoptosis in MG-63 osteosarcoma cells by TGF-β1/PUMA signaling. PUMA is a BH3-only member of the Bcl-2 family and a target of p53-mediated apoptosis.[140] It activates an apoptotic cascade by facilitating Bax activation, causing cytochrome *c* release from the mitochondria, caspase-3 activation, and DNA fragmentation.[141,142] It was also found that miR-29 could target pro-apoptotic PUMA protein and protect against ischemia-reperfusion injury, suggesting that PUMA might be negatively regulated by miR-29. Some studies have reported that PUMA was a direct TGF-β target gene in B cells and that TGF-β induces PUMA to aid induction of the intrinsic cell death pathway.[143] Furthermore, this study also demonstrated the tumor suppressor effect of miR-29 *in vivo*.[144]

miR-29 also affects proliferation through epigenetic control. miR-29 was identified as a negative regulator of *SETDB1*, thereby reducing hepatocellular carcinoma (HCC) cell proliferation *in vitro* and suppressing orthotopic tumorigenicity *in vivo*. Downregulation of miR-29 expression in human HCC contributed to *SETDB1* upregulation by relieving its post-transcriptional regulation. The biological reasons behind this global upregulation of epigenetic regulators in human HCC remain to be elucidated. One possible explanation is that cancer cells are actively dividing and undergoing more dynamic epigenetic reprogramming during cell cycles, and, therefore, they may require increasing amounts of epigenetic regulators to sustain their continuous growth and clonal evolution.[145] From these studies, it is becoming clear that the miR-29 family plays an anti-apoptotic role in several cancers by targeting key regulators in apoptotic pathways.

Metastasis and EMT

Metastasis occurs when cancer cells undergo EMT, a cellular reprogramming event in which epithelial cells revert to a pseudo-stem cell state that is characterized by a loss of polarity and increased migratory behavior.[146] EMT plays a vital role in normal physiology during development as well as in wound healing, but it is also exhibited in cancer metastasis, where a subset of tumor cells disseminates to distant organ sites beyond the site of origination. As a defining signature of stage IV cancer, metastasis is regarded as a major cause of cancer mortality.[147] This is largely because, by the time cancer has metastasized, surgical resection is typically no longer a viable option and metastatic tumors are often refractory to conventional therapies. miR-29 has been implicated to inhibit a number of genes that are involved in EMT and metastasis, even causing the reversion of mesenchymal-epithelial transition (MET) in some cases. However, in contradiction, miR-29 itself has also been shown to directly elicit EMT in a minority of cases.

An initial study in gastric cancers revealed δ-catenin (*CTNND1*) as a target of miR-29.[148] δ-catenin plays an important role in cell adhesion between cells and mediates metastatic signal transduction. Mislocalization of δ-catenin in the cytoplasm or nucleus resulted in the advancement of migration and invasion via regulating Rho GTPase activity and growth factor receptor signaling, implying *CTNND1*'s role as an oncogene.[149,150] A follow-up study in gastric cancer found that the loss of miR-29 contributed to chemoresistance, and overexpression of all three miR-29 family members directly inhibited *CTNND1*, leading to an increased F-actin and Cofilin phosphorylation via activated RhoA.[151] As the F-actin/RhoA pathway is known to influence cell migration, *in vitro* migration assays demonstrated that miR-29c potently inhibited the migratory potential of gastric cancer cells. Consistently, miR-29c inhibited gastric cancer cell metastasis *in vivo* as well.[151] These results confirm that miR-29 controls gastric cancer cell movement by suppressing the catenin-δ pathway. Similarly, another group also reported that miR-29c/b-2 delivery via

recombinant adenovirus decreased gastric cancer cell proliferation and migration *in vitro*.[152]

Dysregulation of miR-29 s also affects cancer cell invasion and migration by the activation of several oncogenic pathways, such as the Wnt/β-catenin pathway. For example, miR-29a overexpression led to decreased β-catenin expression and cell proliferation in non-small-cell lung cancer (NSCLC).[153] miR-29b was shown to inhibit both Wnt/β-catenin and AKT signaling by downregulating *SPIN1* in triple-negative breast cancer (TNBC), and miR-29b overexpression decreased *in vitro* TNBC cell growth, self-renewal, migration, and invasiveness by inhibiting the aforementioned pathways.[154] On the other hand, miR-29 has also been shown as a novel target of β-catenin/Dicer. β-catenin represses Dicer, a key component of the miRNA-processing machinery. Silencing of β-catenin or overexpressing Dicer or miR-29 mimics in highly metastatic ovarian cancer led to significantly reduced migration of cancer cells *in vitro*.[155]

A similar anti-metastatic role of miR-29 has been found in HCC, where miR-29a overexpression in HepG2 cells resulted in a significant decrease in migration *in vitro*. Prior reports have implicated a role for insulin-like growth factor (IGF) and type 1 IGF receptor (IGF-1R) in metastasis.[156] The study went on to validate miR-29a's direct repression of IGF-1R expression.[157] Taken together, miR-29a-mediated inhibition of *IGF1R* exhibited a dual tumor suppressor role in HCC by suppressing HCC cell migration while simultaneously increasing $CD8^+$ T lymphocyte migration.[157] This secondary role of increasing immune cell migration is further covered in the Immunomodulation section.

It is important to note that, in addition to internal reprogramming from EMT, the tumor microenvironment can promote and facilitate metastasis. Different cancers tend to spread to specific organ sites, lending to the seed and soil hypothesis of metastatic microenvironments. Overexpression of ECM components, the major stromal proteins in cancer, is observed in several tumor types, and it contributes to cancer cell progression by the dysregulation of cell adhesion, polarity, and structural remodeling through ECM-modifying enzymes.

miR-29 has been extensively shown to target integrin beta-1 (*ITGB1*).[158] ITGB1 is part of the integrin family of cell surface receptors that have been well established to promote cell growth, migration, and tumor metastasis in a majority of cancers.[159] Integrins typically heterodimerize in alpha/beta pairs and attach to ECM proteins, which serve as tracks for chemotaxis.[160] All three miR-29 family members have been shown to act as potent tumor suppressors in several cancer cell lines, by directly inhibiting *ITGB1* and, thereby, inhibiting migration and invasion (Table S1).

The lysyl oxidase (LOX) protein family functions in covalent cross-linking of collagen and/or elastin in the ECM. Dysregulation of LOXL2 has been reported to correlate with disease progression in several diseases and cancers. Furthermore, LOXL2 interacts with SNAIL1 transcription factor and represses E-cadherin along with inducing EMT. miR-29 has been shown to have a tumor-suppressive role by negatively regulating LOXL2 expression and inhibiting cancer cell migration and invasion in renal cell carcinoma *in vitro*.[117] Moreover, LOXL2 was shown to enhance cancer cell invasion *in vitro* and promote the tumor microenvironment and metastatic niche formation in HCC, where it is activated by HIF-1α/SMAD4 and negatively regulated by miR-26 and miR-29.[161]

Metallomatrix protease 2 (MMP2) is another ECM-modifying enzyme that has been shown to promote metastasis and was found to be a direct target of miR-29. MMP2 plays a critical role in cancer metastasis, and the effects of MMPs on the ECM are well established.[159] Moreover, MMP2 is overexpressed in a variety of primary malignancies, and it is considered an appealing therapeutic target.[162] miR-29c has been shown to target *MMP2* expression, causing decreased pancreatic cancer cell invasion and metastasis *in vitro* and in an orthotopic implantation model in nude mice. Moreover, miR-29 expression was also found to inversely correlate with *MMP2* expression, metastasis, and survival in pancreatic cancer patients.[163] Additionally, the other members of the miR-29 family also regulate *MMP2* expression.[164] Similarly, miR-29b suppresses invasion and metastasis by downregulating MMP2 in HCC, OSCC, and colorectal cancer (CRC) (Table S1).

Similar to the previous reports on miR-29c, miR-29a overexpression was also found to inhibit migration/invasion in pancreatic cancer cell lines,[165,166] but through different mechanisms. One study found that miR-29a inhibited metastatic potential through targeting membrane-bound mucin (MUC1).[166] MUC1 is an epithelial marker of polarized epithelial cells,[167,168] but aberrant overexpression of MUC1 in cancer cells is correlated with an induction of EMT through the Wnt/β-catenin-signaling pathway.[169] miR-29a was shown to directly inhibit MUC1 expression, thereby deregulating signaling pathways promoting EMT.[166] Further studies on the anti-metastatic effects of miR-29 have been linked to its direct inhibition of genes encoding ECM proteins. These related targets are covered more extensively in the following Fibrosis/ECM section.

Finally, GATA3 is a well-studied transcription factor to have potent tumor-suppressive function in various cancers, and it is known to counteract metastasis by inducing MET.[170] In breast cancer cells, GATA3 mediated its tumor-suppressive role through the upregulation of miR-29b.[171] Through an extensive series of experiments, the impact of miR-29b in breast cancer was thoroughly examined, and it revealed that miR-29b inhibition led to a drastic decrease in various epithelial markers, along with increases in mesenchymal markers.[171] Furthermore, miR-29 inhibition *in vivo* resulted in a significant increase in lung metastasis.[171] Most impressively, the study went on to show that miR-29 potently inhibited 15 different targets, including Angiopoietin-like proteins, integrins, *LOXL2*, *MMP*, *PDGF*, *TGFB*, and vascular endothelial growth factor A (*VEGFA*), which are all known to influence metastasis and promote tumor microenvironment progression.[171] In summation, miR-29 functions epistatically

to GATA3 as a potent master regulator to inhibit metastasis in breast cancer.

In contrast to a large body of experimental evidence lending to the anti-metastatic function of miR-29, aberrant expression of the miR-29 family has been reported to induce migration/invasion in certain malignant contexts as well. In pancreatic cancer, miR-29a was reported to act as an oncogene by downregulating tristetraprolin (*TTP*), whose activity is known to be regulated by phosphorylation through the p38 MAPK and ERK-MAPK pathways. Downregulation of *TTP* increased the expression of pro-inflammatory factors and EMT markers,[172] and restoration of TTP decreased cell viability and migration *in vitro* while inhibiting tumor growth and EMT phenotype *in vivo*.[172] Furthermore, miR-29 was also shown to negatively regulate EMT regulator N-myc interactor (NMI) in breast cancer. Increased levels of miR-29 in breast cancer cells inhibited NMI expression, leading to increased cancer cell invasion and promotion of the EMT phenotype.[173] With the absence of NMI, inactivation of GSK3β leads to miR-29 upregulation through unrestricted Wnt/β-catenin signaling.[173] Moreover, in breast cancer, progestin-regulated miR-29 can control progesterone receptor (PR) action by targeting progestin-responsive genes such as *ATP1B1* as well as controlling *PGR* expression itself.[174] Downregulation of miR-29 relieves the repression of *ATP1B1*, allowing it to adjust the progestin response.[174] ATP1B1 limited cell motility, and knockdown of *ATP1B1* led to increased breast cancer cell migration and invasion *in vitro*.[174]

It is perplexing that miR-29 can have such stark contrasting effects on metastasis reported within the same cancer types. These confounding incongruities warrant further studies to better understand these seemingly contradictory results.

Fibrosis/ECM

The ECM is a vital structure that has a dynamic and complex organization, and it can trigger multiple biological activities that are essential for normal organ development and tissue homeostasis. In mammals, the ECM is composed of about 300 proteins known as the core matrisome, which comprises proteins such as collagens; proteoglycans; and glycoproteins such as laminin, elastin, and fibronectin. Dysregulated ECM remodeling leads to many diseases, namely, fibrosis, which also increases the risk of cancer. Fibrosis is a complex process that involves excessive deposition and reorganization of the ECM, leading to EMT.[175] Primarily, fibrosis is mediated by cancer cells that activate fibroblasts, where activated fibroblasts then further perpetuate sustained activation of themselves and other neighboring fibroblasts[175] and lead to fibrotic stromal reaction. Stroma is known to impair drug delivery to the tumor core, promoting tumor progression and metastasis. Consequently, many targeted therapies sought to completely ablate the stroma. However, increasing evidence suggests a greater level of complexity to the story, as the stroma was shown to restrain tumor growth in preclinical studies.[176,177] Recent work has demonstrated that the reductionist approach of all or nothing may not be the appropriate means of targeting the stroma, rather a more subtle degree of crosslinking and stromal density may be key to having a therapeutic impact.[178] With the failure of current stroma-depleting therapeutic strategies, efforts are now focused on developing novel stroma-targeted therapies that appropriately control the fibrotic stroma.

PDGFs and TGF-β are known to induce fibrosis by causing the proliferation of fibroblasts that differentiate into myofibroblasts and produce fibrotic ECM proteins. TGF-β1 is a well-known proinflammatory growth factor that is associated with the pathogenesis of several cancers. TGF-β1 binds to TGF-β receptors (type I and type II) to form an activated hetero-tetramer with serine-threonine kinase activity, which phosphorylates downstream transcription factors SMAD2 and SMAD3. Upon phosphorylation and activation, pSMAD2/3 form a heterogeneous complex with SMAD4, translocate into the nucleus, and directly regulate target gene expression. Several of these target genes include ECM proteins, such as *COL1A1*, *COL3A1*, and *TIMP1*, as well as about 60 other ECM-related genes.

Several studies have implicated that miRNAs function as potent regulators of the ECM, and, in particular, the miR-29 family has been well established as a potent anti-fibrotic miRNA. Initial studies found that miR-29 restoration quelled fibrosis and ECM protein production in the context of numerous organs, including cardiac,[6] liver,[179] lung,[10] and kidney[180] fibroses. Moreover, TGF-β signaling is responsible for the decreased miR-29 expression during fibrosis. Hence, targeting the miR-29 family and TGF-β may be an effective strategy for the treatment and management of fibrosis.[181,182] The interaction between the miR-29 family and TGF-β was revealed in lung and cardiac fibroses.[6,8] After treatment of TGF-β1 on miR-29 knockdown IMR-90 cells,[8] certain genes were confirmed to be upregulated by TGF-β, and these genes mainly consisted of miR-29-predicted targets, such as collagens. Furthermore, the stimulation of TGF-β1 and knockdown of miR-29 exhibited similar behavior in lung cells, and they led to the upregulation of specific fibrotic genes, such as *COL1A1*, *COL3A1*, and *COL1A2*. Various laminins, integrins, MMPs, and ADAMs are upregulated with miR-29 downregulation as well, but not with TGF-β1 stimulation.[8] Therefore, these observations show that the miR-29 family regulates gene expression via TGF-β-dependent and -independent signaling pathways.

More recent studies revealed how miR-29 mediates TGF-β1 pathways by targeting a variety of ECM network genes. For example, Hsp47, a collagen-binding protein and collagen-specific chaperone, is a hub of the ECM network and controls the tumor microenvironment by the deposition regulation of several ECM proteins. A transcription network analysis showed that Hsp47 expression was activated during breast cancer development and progression, while Hsp47 silencing reprogrammed human breast cancer cells to form growth-arrested and/or non-invasive structures in 3D cultures, and, moreover, it restricted tumor growth in xenograft assays by decreasing the deposition of collagen (COL1A1 and COL4A1) and fibronectin (FN1). Co-expression network analysis showed miR-29b and -29c were inversely correlated with Hsp47 expression and other ECM network genes. In summary, in an *in vitro* and *in vivo* breast cancer study,

miR-29 mediated TGF-β1-induced Hsp47 expression, promoting breast cancer by increased ECM deposition.[183] An interesting study found that knockdown of miR-29 enhanced the ionizing radiation-induced expression of type I collagen through the TGF-β-Smad3-signaling pathway in irradiated cells. Inhibition of TGF-β-Smad3 signaling blocked the significant loss of miR-29, and miR-29 overexpression inhibited the ionizing radiation-induced expression of type I collagen, suggesting that miR-29 may be an important regulator of radiation-induced fibrosis (RIF).[184] However, further studies are required to dissect the role of miR-29 in molecular mechanisms associated with radiation.

Moreover, miR-29 downregulation leads to increased ECM protein expression levels of collagens and laminins, as observed in a number of cancers. Based on miRNA-mRNA covariation and sequence-based target predictions, miR-29a/c were identified as novel regulators of LAMA2, an ECM protein associated with poor prognosis in posterior fossa (PF) ependymoma. Decreased miR-29a/c expression correlated with elevated LAMA2 expression are features of PF ependymoma post-transcriptional regulation.[185] Moreover, the miR-29 family was identified to have one of the strongest interactions between DEGs in an interaction network derived from gene expression profile data (GEO: GSE12452) of nasopharyngeal carcinoma (NPC) tissue specimens along with an miRNA-sequencing dataset (GEO: GSE14738). *COL3A1*, *COL1A1*, *COL4A1*, and *COL5A2* were found to be regulated by several miR-29 family members, suggesting that miR-29 may be related to the development of NPC by the regulation of these genes involved in ECM-receptor interaction.[186]

Similarly, in a study aiming to explore the regulatory mechanism of CRC and potential novel biomarkers for screening, COL1A1 was predicted to be targeted by miR-29 from examining the protein-protein interaction network (PPI) from microarray data (GEO: GSE44861).[187] In 2013, a study found that the reduction of miR-29 expression led to increased cisplatin resistance of ovarian cancer cells, partly through upregulating the expression of ECM components such as COL1A1 *in vitro* and *in vivo* and also via increasing the activation of ERK1/2 and inactivation of GSK3β. Therefore, the downregulation of miR-29 in ovarian cancer cells manipulates the surrounding ECM to enhance survival signal transduction upon cisplatin treatment. Ectopic expression of miR-29 alone or in combination with cisplatin effectively reduced tumorigenicity of CP70 ovarian cancer cells *in vivo*, suggesting that miR-29 overexpression may have therapeutic implications as a potential sensitizer to cisplatin treatment by remodeling the ECM.[188] Furthermore, a recent study displayed the role of the miR-29 family in preserving cardiac health in the regulation of age-dependent increases of oxidative stress and cardiac fibrosis. It was found that the miR-29 family is one of the most upregulated miRNAs during aging, and its increase induces the downregulation of known targets such as collagens, DNMTs, and 5′methylcytosine (5mC). In addition, under hypoxic conditions, miR-29a/-29b downregulation was found to be responsible for collagen deposition and fibrosis. Therefore, the miR-29 family upregulation may play a role as an endogenous mechanism against cardiac fibrosis and hypertrophy in age-dependent cardiac damage.[189]

Pancreatic cancer is notoriously known to be one of the most fibrotic cancers, and its associated stroma has been shown to play an integral role in tumor progression and resistance to therapy.[190] Pancreatic ductal adenocarcinoma (PDAC) is unique in that the degree of intratumoral fibrosis is so extreme in many cases that the bulk of the tumor is made up of the stromal compartment.[190] Hence, pancreatic cancer frequently serves as a surrogate in studying cancer-stroma interactions. In PDAC tumors, pancreatic stellate cells (PSCs) are mainly responsible for the production of ECM proteins and the dense fibrotic stroma surrounding the tumor bed. Cancer cells secrete a slew of pro-inflammatory growth factors and cytokines, including TGF-β1, which activate PSCs. In turn, activated PSCs and fibroblasts produce pro-inflammatory growth factors and chemokines that act in an autocrine fashion to maintain their sustained activity and produce fibrotic stromal ECM proteins.[191] In recent years, we found a significant downregulation of miR-29a and miR-29b in TGF-β1-activated PSCs, fibroblasts, as well as cancer-associated fibroblasts (CAFs).[45] Furthermore, we found that miR-29 was significantly downregulated in PSCs of a KRAS mutant genetically engineered mouse model of pancreatic cancer (KC) as well as in PDAC patient biopsies via miRNA *in situ* hybridization.[45] Restoration of miR-29a or -29b in activated PSCs caused a drastic decrease in the expression of various ECM proteins, and it reduced cancer colony growth in co-culture.[45] Taken together, our studies indicate that miR-29 may have therapeutic relevance beyond just the cancer cells themselves. Further studies may reveal miR-29 to be a potent anti-tumorigenic agent in other stromal cells as well.

miR-29 has also been observed to target genes outside of the ECM network to provide protective effects against fibrosis. Arrestin beta 1 (ARRB1) is a member of the arrestin and beta-arrestin protein family thought to participate in agonist-mediated desensitization of G-protein-coupled receptors and cause dampening of cellular responses to stimuli, such as hormones, neurotransmitters, or sensory signals. In a 2017 study, miR-29 along with miR-652 were indicated as biomarkers in liver fibrosis. In a luciferase reporter-based target validation assay, *ARRB1* was identified as a target gene of these two miRNAs in regulating the onset and progression of liver fibrosis. Decreased expression of ARRB1 in addition to Th17, IL-17A, and IL-22 was observed in miR-29a-transfected CD4+ T cells isolated from the spleen of normal mice. Moreover, miR-29 overexpression led to decreased ALT, AST, Th17, and ARRB1 levels *in vivo*, suggesting the protective effects of miR-29 overexpression in liver fibrosis.[192]

The wider scope of these studies demonstrates anti-fibrotic activity of miR-29 and its role in regulating a number of ECM proteins, and it suggests an important role in the homeostasis of the ECM. A considerable amount of experimental evidence has shown that restoring downregulated miR-29 expression with synthetic miRNA mimics may pose a suitable strategy to overcome fibrosis. Though, this

strategy still needs to be validated in advanced preclinical studies and in clinical settings.

Angiogenesis

The vascular system provides a vital channel to the delivery of nutrients and oxygen, and it has been shown to be crucial for tumor growth.[193,194] Consistently, cancer cells exploit the process of angiogenesis to generate new blood vessels to redirect nutrients and oxygen for their growth.[195] In addition to supplying provisions to cancer cell growth, blood vessels also provide an effective conduit for metastasis.[195] Therefore, numerous efforts have been underway to target angiogenesis in various cancers.[196]

miR-29 has been well implicated in the process of angiogenesis as an anti-angiogenic agent by potently inhibiting VEGFA.[197,198] VEGFA is a functionally dominant member of the VEGF family of growth factor proteins that is typically secreted and acts on endothelial cells to promote their growth, migration, and angiogenic properties.[199] As expected, it has been reported that various cancers overexpress VEGF,[200] and a number of studies have shown all three miR-29 family members to be potent tumor suppressors through directly targeting VEGFA.[171,201–206]

However, beyond the canonical downregulation of *VEGF* via 3′ UTR binding, a couple of other studies have indicated new mechanisms of regulation involving miR-29 sponging and a role in cancer-stroma cross-talk. An interesting report on osteosarcoma claimed a novel mechanism by which the miR-29-binding site within the 3′ UTR of *IGF1* functions as a competing endogenous RNA for miR-29.[207] By effectively sponging miR-29, authors showed an increase in *VEGFA* mRNA expression.[207] This study stands alone in this claim, but this newly proposed mechanism of competitive binding warrants further studies nonetheless.

More recently, miR-29 has been proposed to be involved in a paracrine cross-talk mechanism between cancer-secreted IGF2 on VEGF expression in CAFs.[203] Initial findings indicated that there was a concordant elevated expression of IGF2 in cancer cells with an increase in VEGF expression in CAFs, which conferred an unfavorable prognosis in esophageal squamous cell carcinoma.[203] Utilizing predictive algorithms, miR-29c and miR-127 were identified to have some of the highest predictive scores for *VEGF* 3′ UTR-binding sites, and they were also found to be some of the most significantly downregulated miRNAs in IGF2-treated CAFs.[203] Follow-up functional studies with both miRNAs revealed that only the overexpression of miR-29c led to a significant and consistent downregulation of VEGF in CAFs.[203]

Furthermore, miR-29 expression has even been shown to correlate with levels of tumor vasculature in clinical samples. In a study looking at the expression levels of various miRNAs in endometrial patient biopsies, miR-29b was found to be significantly downregulated in tumor samples compared to normal endometria and had a significant inverse correlation with the degree of tumor vascular invasion.[208] In addition, low miR-29 expression levels conferred poorer survival.[208]

Collectively, an insurmountable number of studies demonstrate that miR-29 effectively targets *VEGFA*, which is an appealing therapeutic target in cancer. In fact, several clinical trials have been conducted or have been ongoing in an attempt to target VEGFA in various cancers (ClinicalTrials.gov: NCT01351415, NCT01863693, and NCT01239732). However, anti-angiogenesis treatment alone does not eliminate cancer completely and requires a combination therapeutic approach.[196] Perhaps, miR-29 may also serve as an alternative and effective means of targeting VEGFA in combination with other therapies.

Immunomodulation

miR-29 has been shown to play a role in various immune-related aspects, from adaptive and innate immune responses[107] to viral pathogenesis.[89,209] Although miR-29 has not been studied extensively in cancer immunity, a few studies have started to reveal its role in the context of cancer immunity.

B7-H3 is a potent immune checkpoint inhibitor that is known to be overexpressed by cancer cells and causes T cell suppression.[210] Unsurprisingly, B7-H3 has become an appealing therapeutic target in cancer.[210] In fact, a monoclonal antibody targeting B7-H3 is already undergoing clinical trials (ClinicalTrials.gov: NCT02475213). Moreover, a study investigating various solid tumors found all three miR-29 family members to be significantly downregulated in multiple cancers compared to normal tissues and inversely correlated with B7-H6 expression.[211] The *B7-H6* miR-29-binding site was validated, and overexpression of miR-29a led to a potent inhibition of B7-H6 expression.[211] Although these findings convincingly demonstrate a potential use of miR-29 as a therapeutic inhibitor of B7-H6, further studies are needed to provide evidence of functional significance *in vivo*.

Another interesting immune-related mechanism of miR-29 was discovered in liver cancer, where miR-29a overexpression in HCC cells downregulated cancer cell migration while simultaneously increasing migration of $CD8^+$ T lymphocytes *in vivo*.[157] miR-29 overexpression downregulated IGF-1R in HCC cells, which led to an increased production of chemokine ligand 5 (CCL5).[157] CCL5 is important for chemotactic movement of $CD8^+$ T lymphocytes,[212] and this was of particular significance as patients with high CCL5 exhibited better survival rates due to increased immune cell infiltration into the tumor.[157] Albeit the mechanism was through indirect effects, these results still provide additional evidence of miR-29's immunomodulatory function in cancer.

Beyond miR-29's intrinsic function within cancer cells, miR-29 has also been shown to elicit an inflammatory response extrinsically. A subset of immune cells has been known to detect exogenous RNA through Toll-like receptors (TLRs) and trigger an inflammatory immune response.[213,214] As miRNAs had been reported to be released

by cells to function extracellularly, one study sought to determine if secreted miRNAs interact with TLRs in the context of cancer.[215] Indeed, miR-29a was shown to be secreted and co-immunoprecipitated with TLR7.[215] Furthermore, miR-29a was found to co-localize with macrophage TLR7 and TLR8 *in vivo*.[215] Ultimately, miR-29 binding to TLRs led to nuclear factor κB (NF-κB) activation and subsequent cytokine production that enhanced the metastatic potential of cancer.[157] This proved to be a novel means by which miRNAs can function in an extrinsic manner beyond their canonical target mRNA 3′ UTR-binding mechanisms.

Only a handful of reports have demonstrated miR-29's role in cancer immunomodulation, and, among these few studies, all were within the context of cancer cells. It has yet to be determined whether miR-29 plays an intrinsic role within immune cells themselves in relation to cancer pathogenesis. Certainly, miR-29's role in immune cells outside of the tumor setting has been studied. For example, systematic screening of >100 individual miRNAs in helper T cells revealed miR-29 as a critical regulator of the IFN-γ pathway through the direct targeting of two critical transcription factors, T-bet and Eomes.[216] T cells are known to be involved in the cancer immune response,[217] and, therefore, it is not hard to imagine that miR-29 could have a functionally important role in cancer immunity as well. The investigation of miR-29 expression status and function in the context of intratumoral immune cells is of particular interest and remains an open area of research.

Clinical Findings and Prognostic Implications

The majority of accounts have reported miR-29 to have a tumor suppressor function in various cancer types. However, compared to the number of mechanistic studies of miR-29 in cancer, there are noticeably fewer studies interrogating the clinical ramifications of miR-29 expression for patient overall survival and outcomes. Herein, we touch on the current state of miR-29 as a potential prognostic marker.

In lung cancer, a study profiling the miRNA expression levels in tumor biopsies found miR-29b to be one of the most significantly downregulated miRNAs.[218] Consistently, another study sought to define molecular features that distinguish lung squamous cell carcinoma versus adenocarcinoma, and the researchers found that all three miR-29 family members were more heavily downregulated in squamous cell carcinoma, with miR-29a as one of the most prominently downregulated miRNAs.[219] Even though miR-29 was significantly downregulated in lung tumors, these studies highlighted various other miRNAs as having a more prominent effect in predicting overall survival.[218,219] However, a more recent report specifically looking at the impact of miR-29 expression level on overall survival and relapse-free survival in lung cancer patients found that patients with tumors expressing high miR-29 led to significantly better survival outcomes.[220] Oftentimes, all three miR-29 family members were found to negatively correlate with prognostically unfavorable pulmonary neuroendocrine tumor grades. In contrast to miR-29 expression levels in tumors, two independent groups profiled circulating miRNA levels, and they found that higher levels of miR-29 in plasma conferred lower survival rates.[221,222] Although tumor expression and liquid biopsies are not one in the same, these results are still confounding.

These incongruent results are found in various lymphoma-related studies as well. In a single study, miR-29c was found to be upregulated in AML patients and was associated with a higher risk of relapse.[223] In stark contrast, miR-29a was found to be significantly decreased in the bone marrow samples of 106 AML patients.[224] Furthermore, high miR-29a expression was associated with more favorable prognosis, increased survival, and relapse-free survival.[224] Concordant with these results, another study found both miR-29a and miR-29b expression levels to be significantly downregulated in myeloid leukemia and inversely correlated with anti-apoptotic gene *BCL2* and *MCL1* expression levels.[225] The authors noted that lower levels of *BCL2* and *MCL1* had been reported to confer better overall survival in myeloid leukemia patients. However, the study did not demonstrate that miR-29 expression correlated with survival within their patient samples.[225]

There are limited reported studies regarding the prognostic value of miR-29 in other cancer types. Nevertheless, many of these studies seem to consistently indicate that high miR-29 expression leads to increased survival. In the case of mantle cell lymphoma, patients with significantly decreased miR-29 levels had shorter survival and had an inverse correlation with CDK6.[105] Similar findings were reported in gastric cancer,[226] melanoma,[227] and serum miR-29 in high-grade glioma,[228] where increased miR-29 indicated better survival.

From the existing evidence, the prognostic value of miR-29 expression is not clear and further studies are needed. Yet, most of these biomarker studies simply dichotomize patient cohorts based on high versus low miR-29 expression. Perhaps, further studies with a higher degree of scrutiny in patient stratification will better clarify and rectify these mixed results.

Discussion

Since the discovery of the first miRNA, the area of miRNA research has rapidly expanded and moved beyond basic biology into therapeutic use. Just within the past two decades, >2,000 patent documents related to miRNAs have been published.[229] Nonetheless, the development of miRNA therapeutics lags behind many other forms of treatment, including RNAi-based drugs.[230] Furthermore, recent setbacks were made when the first miRNA replacement therapy clinical trial, testing the therapeutic effect of miR-34 (MRX34) in cancer, was halted at phase I due to immune-related side effects.[231] However, some arguments have been made that the adverse events of the MRX34 clinical trials were not due to miR-34 itself, but rather caused by the method of delivery (e.g., liposomes) or immunostimulation induced by double-stranded RNA.[231,232]

Nevertheless, the promise of miRNA-based therapies in their ability to simultaneously target multiple disease-related pathways has remained appealing, as indicated by numerous therapeutic products

still actively being pursued within the pipelines of several biotech companies.[230] In fact, the miR-29 mimic MRG-201 is currently being tested in phase I clinical trials via intradermal injection (ClinicalTrials.gov: NCT02603224). Recently, a major milestone has been accomplished in the area of small RNA therapeutics, with the advent of the first FDA-approved siRNA treatment for a rare neurological disease, hereditary transthyretin-mediated amyloidosis (hATTR). The siRNA, patisiran, functions by inhibiting abnormal transthyretin (TTR) to reduce amyloid deposits and significantly improves symptoms associated with hATTR, including reflexes and motor strength.[233] The delivery method in the clinical study also utilized a lipid nanoparticle to package and deliver the siRNAs, but it is important to note that there were complications associated with patisiran infusion.[233] However, with the administration of anti-inflammatory drugs and antihistamines alleviated these issues.[233] As new and improved modes of gene delivery are developed and tested,[234–236] it may be just a matter of time before the therapeutic delivery of miR-29 will be tested in cancer.

Although miR-29 may seem to show a high degree of promise as a potent tumor suppressor, the minority of studies reporting miR-29 as an oncogene are still grounds for circumspection and must be strongly taken into consideration (Figure 2B). The opposing effects of miR-29 reported even within the same cancer types, such as breast cancer[174,183,237,238] and pancreatic cancer,[158,163,165,166,172] are confounding.

In breast cancer, two studies took a systematic approach of assessing miR-29 status in breast cancer, revealing similar results of miR-29 as a tumor suppressor miRNA.[174,183] While Zhu and colleagues[183] conducted a network analysis of gene co-expressions in hundreds of clinical breast cancer samples, Duhachek-Muggy and Zolkiewska[237] profiled a panel of 50 breast cancer cell lines. Although overlapping breast cancer cell lines (BT549 and MDA-MB-231) were utilized in validating downstream mechanisms in both studies, only Zhu et al.[183] showed functional impact of miR-29b and -29c in reducing colony formation. In two separate studies investigating the role of miR-29 in the context of breast cancer cell response to progesterone receptor activation, Cochrane et al.[174] postulated that miR-29a and miR-29b function as tumor promoters by inhibiting ATP1B1 expression. In direct contrast, Cittelly et al.[238] demonstrated that miR-29 inhibition in T47D and BT474 cells using antagomirs leads to increased growth in 3D colony formation assays. Further *in vivo* validation in an orthotopic mouse model demonstrated mir-29 inhibition leads to greater tumor growth and metastases. Although Cittelly and colleagues[238] utilized the same breast cancer cell line, T47D, they found contradictory results. However, Cochrane et al.[174] only conducted an *in vitro* viability assay in the T47D cell line, and they did not verify the impact of miR-29 *in vivo*.

In pancreatic cancer, four studies found that miR-29 exhibited a tumor-suppressive function,[158,163,165,166] as opposed to a single study that showed entirely opposite effects.[172] Four studies functionally verified the tumor-suppressive properties of miR-29 by transfecting miR-29 mimics in pancreatic cancer cells and subjecting them to various *in vitro* assays to assess migration/invasion, anchorage-independent growth, and proliferation.[158,165,166] Two studies went on to test the functional effect of miR-29 *in vivo*, and they consistently found that miR-29 suppressed tumor growth.[158,163] In direct contrast to these studies, Sun and colleagues[172] found that miR-29a functions as a tumor promoter in various *in vitro* assays as well as *in vivo*. Further confusion arises when multiple mechanisms are proposed to explain the anti-tumor effects of miR-29 within the same cancer. For example, miR-29 was shown to have anti-metastatic potential in pancreatic cancer, yet all three reports offered alternative hypotheses for which miR-29 target downregulation leads to reduced migration/invasion (*MMP2*, *MUC1*, *ITGB1*).[158,163,166]

Some of the discrepancies of these studies can be explained by subtle differences in methodologies and the utilization of different cell lines. For example, although Cochrane et al.[174] and Cittelly et al.[238] found opposite results for miR-29 in the same cell line, T47D, Cittelly tested the impact of miR-29 in a particular $CD44^+$ cancer stem cell subpopulation.[238] However, there are examples in which using the exact same methodology and cell lines yielded contradictory results. Sun et al.[172] found miR-29a to be increased in Panc-1 and BxPC-3 cell lines compared to a normal pancreatic epithelial line. In direct contrast, Tréhoux et al.[166] and Kwon et al.[165] consistently found its significant downregulation in various pancreatic cancer cell lines, including MIA PaCa-2, Panc-1, and BxPC-3, compared to normal pancreatic epithelial cell lines. Further discrepancies arise whereby Sun et al.[172] overexpressed miR-29a mimic similar to both Tréhoux et al.[166] and Kwon et al.,[165] only to find the exact opposite results of increased cell proliferation and migration. A potential explanation for these confounding results could be explained by recent reports demonstrating heterogeneity between the same cell line from different labs.[239] 27 strains of the same well-utilized cell line MCF7 demonstrated immense genetic diversity and a large variability in drug response.[239] By speculation, the opposing results of these studies could be explained by the diversity that resides within the same cell lines. Nevertheless, these incongruities demand the need for further studies and greater meticulous measures in validating cell lines and reagents to better understand these contradictory results.

Genomic heterogeneity is a well-known feature of cancer exists between tumors of different patients and is even evident within the same tumor.[240] In recent years, there has been a paradigm shift toward classifying cancers based on genetic alterations rather than in the context of anatomical location. In fact, Pembrolizumab was the first recently FDA-approved cancer therapy on the basis of the patients' tumor gene expression profile instead of the cancer's originating tissue type. As miRNAs function in a context-dependent manner,[241–244] the discrepancies of miR-29 function and alternative mechanisms of action may be due to a lack of contextualizing miR-29 against specific genetic backgrounds. This is particularly relevant given that several different mechanisms of regulating miR-29 expression have been reported. Common mechanisms of miR-29

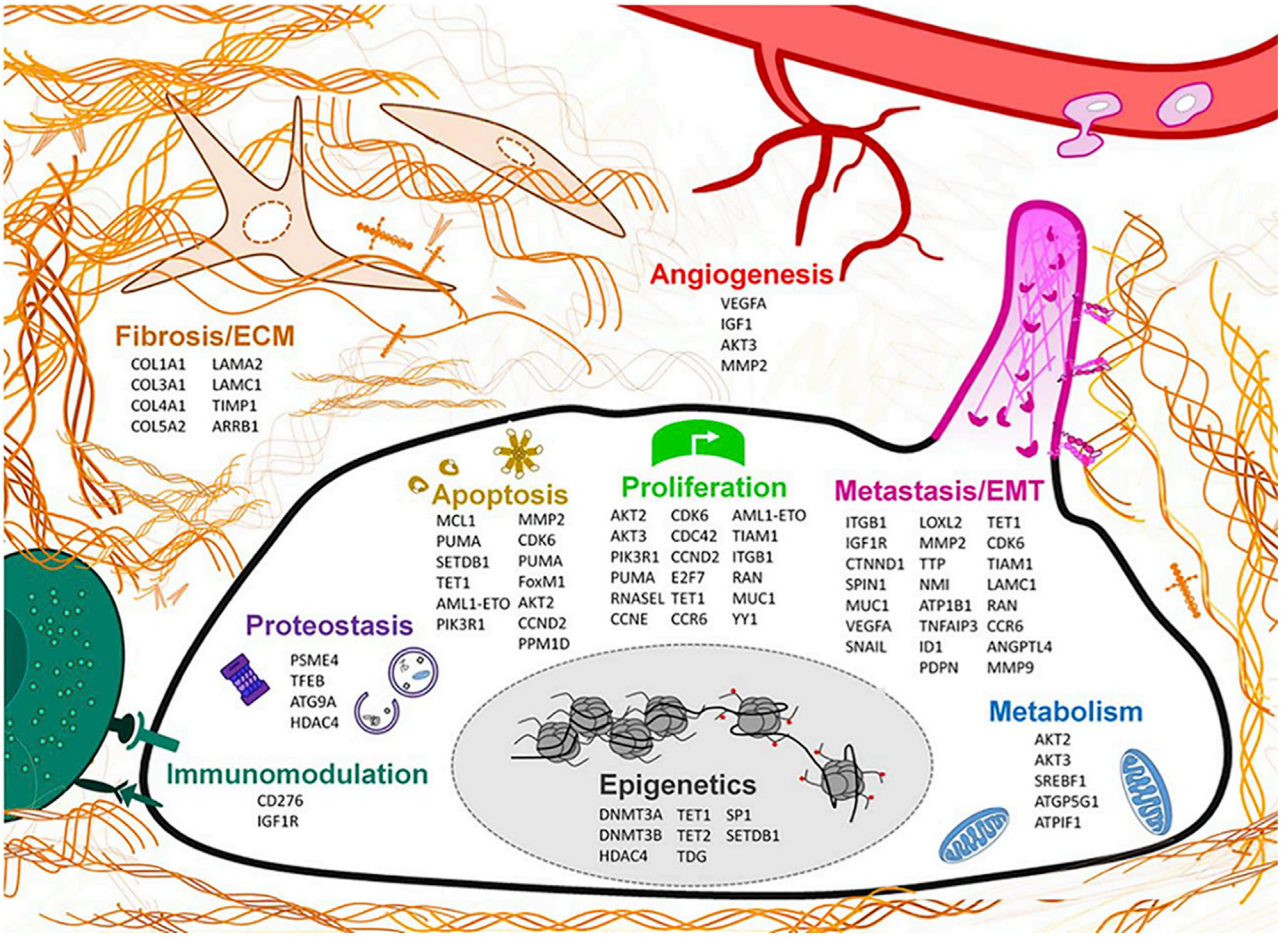

Figure 3. miR-29 Targets in the Mechanisms of Cancer

Schematic illustration depicts the reported targets of miR-29 involved in epigenetics, proteostasis, metabolism, proliferation, apoptosis, metastasis, fibrosis, angiogenesis, and immunomodulation.

dysregulation in cancers have been identified, such as MYC directly suppressing miR-29 expression in various cancers.[125,245,246] However, a few context-specific dysregulations of miR-29 expression have been demonstrated as well.

Given the anti-inflammatory role of miR-29 in fibrosis, it is not surprising that TGF-β1-SMAD signaling has been shown to directly downregulate miR-29 expression.[7–12] Consistently, restoration of miR-29 in activated pancreatic fibroblasts has been shown to reduce cancer cell proliferation in co-culture systems, and miR-29 downregulation in these fibroblasts was found to be SMAD3 dependent.[45] These findings coincide with similar effects reported in ovarian cancer, where TGF-β1 has been shown to elicit pro-tumorigenic function by simultaneously downregulating miR-29.[247] However, the majority of reports demonstrating TGF-β1-SMAD3/4-mediated miR-29 suppression have been in the context of fibroblasts and fibrosis. Thus, this mechanism of miR-29 dysregulation may be a more fibroblast-specific phenomenon.

A notable example of miR-29 dysregulation involves NF-κB and YY1. Both NF-κB and YY1 have been shown to be vital in the process of regulating myocyte differentiation.[248,249] In rhabdomyosarcoma, miR-29 has been found to be a tumor suppressor, where the molecule downregulates several cell cycle regulatory genes.[128] Unsurprisingly, a rhabdomyosarcoma-specific mechanism of miR-29 dysregulation was found to be mediated by an NF-κB-YY1-miR-29-signaling axis, whereby YY1, under the regulation of NF-κB, directly binds to the miR-29 promoter site, and it modulates its expression epigenetically via a negative feedback loop mechanism and by closing the accessibility of chromatin modulation at the miR-29 promoter site.[250]

Another context-specific example of miR-29 dysregulation involves the transcription factor GATA3. Specifically, GATA3 has been shown to be a tumor suppressor in breast cancer by maintaining luminal cell differentiation and inhibiting metastasis.[251,252] Accordingly, the loss of GATA3 has been shown to be associated with poor prognosis, and it was found to be mutated in several cases of breast cancer.[253,254]

In elucidating the mechanism by which GATA3 functions as a tumor suppressor, miR-29 was found to be a critical downstream target of GATA3.[171] Specifically, miR-29b was found to be the most differentially upregulated miRNA by GATA3, where GATA3 directly binds to the promoter of miR-29.[171] Finally, miR-29 was found to be a dominant effector of GATA3, where the inhibition of miR-29 was found to be sufficient for ablating the GATA3 tumor suppressor function.[171]

Most of the current miR-29-related literature in cancer frequently generalizes the function of miR-29 within the framework of organ-based stratification, without acknowledging the genetic background of the cells and biopsies. This may be due to utilizing a traditional, reductionist approach in biology. Factoring in many genetic variables is a daunting if not impossible task. However, recent advancements in systems biology and high-throughput technologies, such as proteogenomics, next-generation sequencing, single-cell sequencing, and functional genomics, have allowed the field to gain a more refined insight into global gene regulation, as well as having provided a more systematic approach to testing the function of genes on a large scale. Furthermore, the accumulation of large genomic databases (e.g., Human Cell Atlas, TCGA, The Human Protein Atlas, Project Achilles, DepMap, PRISM, and Cancer Cell Line Encyclopedia) has allowed for a greater degree of resolution in studying the heterogeneity associated with cancer.

Indeed, some systematic approaches have been conducted for miR-29. For example, miRNA expression across 10 cancer types was analyzed in TCGA database to determine commonalities in miRNA-target networks.[64] Notably, "miR-29a was generally downregulated…in tumors of most cancer types as compared with representative normal samples."[64] Still, other analyses utilizing comprehensive, unbiased approaches find similarly consistent results of miR-29 as a tumor suppressor.[255,256] With time, further methodical studies employing these techniques and utilizing big data may provide a more auspicious approach to better understanding miR-29's function in the context of cancer, and they may clarify its disputed role as tumor suppressor versus tumor promoter.

The multifaceted function of miR-29 casts doubts in claiming it as a panacea for cancer. It seems that miR-29's dichotomous role as a tumor suppressor versus oncogene may be context dependent. However, at the current moment, miR-29 has been predominantly shown to function as a tumor suppressor in the majority of publications, with >85% of all miR-29-cancer-related studies (Figure 2B) demonstrating miR-29 inhibiting various cancer-related targets (Figure 3). Future studies are of interest to see if investigating the function of miR-29 in a more comprehensive and systematic manner will resolve these disputes and elucidate a more refined perspective on miR-29's role in cancer.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one table and can be found with this article online at https://doi.org/10.1016/j.omto.2018.12.011.

AUTHOR CONTRIBUTIONS

J.J.K. and J.K. conceived this work. J.J.K. and T.D.F. generated all figures. J.J.K., T.D.F., and S.D. manually curated the literature, organized the data, and wrote the manuscript.

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