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# MICRORNAS IN DEVELOPMENT AND DISEASE

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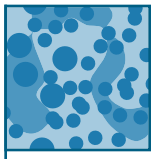

Sayed D, Abdellatif M. MicroRNAs in Development and Disease. *Physiol Rev* 91: 827–887, 2011; doi:10.1152/physrev.00006.2010.—MicroRNAs (miRNAs) are a class of posttranscriptional regulators that have recently introduced an additional level of intricacy to our understanding of gene regulation. There are currently over 10,000 miRNAs that have been identified in a range of species including metazoa, mycetozoa, viridiplantae, and viruses, of which 940, to date, are found in humans. It is estimated that more than 60% of human protein-coding genes harbor miRNA target sites in their 3′ untranslated region and, thus, are potentially regulated by these molecules in health and disease. This review will first briefly describe the discovery, structure, and mode of function of miRNAs in mammalian cells, before elaborating on their roles and significance during development and pathogenesis in the various mammalian organs, while attempting to reconcile their functions with our existing knowledge of their targets. Finally, we will summarize some of the advances made in utilizing miRNAs in therapeutics.



## I. INTRODUCTION

### A. The Discovery of MicroRNAs and Their Significance

In 1993, discovering that lin-4 is a 22-ribonucleotide-long molecule that negatively regulates lin-14, not only identified the first microRNA (miRNA), but also revealed their functional relevance (341). Lin-4 is one of the heterochronic genes that are involved in temporal regulation of "larva-to-adult switch" in *Caenorhabditis elegans* via inhibiting the expression of lin-14 and lin-28 (10). Accordingly, loss of lin-4 results in inappropriate "reiteration of cell lineages" during later developmental stages, which is a sign of perturbed developmental timing (81). After cloning the lin-4 gene, it was surprising to find that it had no substantial open reading frame, but did generate two RNA products, ~61 and 22 nucleotides (nt) long, which are now recognized as the premature and mature forms of lin-4, respectively (341). The mature form is complementary to repeat sequences in the 3′-untranslated region (UTR) of lin-14 mRNA (341) via which it inhibits its translation without affecting its stability (461). A similar mechanism was later ascribed to let-7 and its regulation of a set of genes that included lin-14, lin-28, lin-41, lin-42, and daf-12 in *C. elegans*, where if over- or underexpressed resulted in premature development or reiteration of cell lineages (506). These findings reveal two aspects of miRNA's unique functionality: *1*) precise regulation of the timing of a cellular event via *2*) synchronous inhibition of a cadre of genes that are functionally interdependent, thus operating as an efficient molecular switch. This mode of function circumvents the need for transcriptional regulation of individual genes and is, thus, potentially faster and more energy efficient, consistent with what Beyer et al. referred to as translation regulation "on demand" (40). Furthermore, posttranscriptional coregulation of functionally related genes is well supported by two observations: *1*) the positive correlation between mRNA and protein abundance of functionally related genes, but not those of whole cell or separate cellular compartments (40); and *2*) mRNA of functionally related genes often possess similar decay rates (655). miRNAs have the necessary functional versatility and specificity to explain both these cellular attributes.

As we were reviewing the vast literature regarding the role of miRNAs in mammalian organogenesis and pathogenesis, we continually searched for patterns that could explain the functional relevance of these posttranscriptional regulators.

One of the more reproducible patterns that was seen during the development of various organs was reminiscent of lin-4 in *C. elegans*, where over- or underexpression of a miRNA either accelerated or delayed differentiation of cells, indicative of its regulation of developmental timing. Some of the examples that will be encountered in this review include the role of miR-27 in myogeneis (57), the role of miR-124 (101) and miR-9 in neurogenesis (558, 711), the role of miR-30 in nephrogenesis (5), and the role of miR-17~92 in lung development (390).

## B. The Expression and Processing of miRNAs

To date, 940 human miRNAs have been identified, which are annotated and catalogued in a searchable Web-based miRNA database known as miRBase (217, 218). The first group of mammalian miRNAs was identified by means of large-scale cloning and sequencing of the mature forms (327, 334, 340). To accurately differentiate between miRNAs and other small RNA species, such as siRNAs, Ambros et al. (11) defined a set of expression and biogenesis criteria that were used for that purpose. The expression criteria included the ability to detect an ~22 nt RNA transcript by conventional Northern blotting, or an equivalent method, and in a cDNA library of size fractionated RNA. The biogenesis criteria included the presence of the ~22 nt sequence in one of the arms of a putative stem-loop secondary structure that had to include "at least 16 bp involving the first 22 nt of the miRNA and the other arm of the hairpin" (11). In addition, the sequence of the secondary structure had to be phylogenetically conserved and accumulate in cells upon loss of the processing enzyme Dicer. The structural aspects of these criteria were used in a "training set" for computer software to generate algorithms for computational prediction of miRNAs within the genome (reviewed in Ref. 33). The data from that information were then further examined in an attempt to delineate the transcriptional units of those genes (509). Out of 232 mammalian miRNAs analyzed, ~40% existed within introns of coding RNA, 10% within introns of noncoding RNA (ncRNA), and 13% within exons of ncRNA, in addition, a small portion of miRNAs exist within exons (mainly 3′ untranslated regions) of coding "host" genes, some of which are expressed from the same transcriptional unit (509, 625). The remaining miRNAs are intergenic, with mostly undefined transcriptional units as of yet. For that purpose, computational tools were developed for prediction of miRNA core promoters (271, 715). These applications revealed that miRNA genes have features common to RNA polymerase II-dependent promoters, as well as unique sequence motifs. Otherwise, there are only a handful of studies that experimentally examined the transcriptional regulation of individual miRNA promoters. Examples include the mouse miR-1–1 and miR-1–2 promoters that are regulated by serum response factor (SRF), myoD, and muscle enhancer factor 2 (Mef2c), also thought to cotranscribe miR-133a-2 and miR-133a-1, respectively, as part of bicistronic transcripts (713), and the miR-206 promoter, which binds myoD and myogenin (501), among a few others (141, 459). Additionally, a fair number of miRNA genes exist within areas of Alu repeats that are transcribed by RNA-polymerase III-dependent promoters (54).

The primary transcript of a miRNA (pri-miRNA) gene encompasses a stem-loop precursor (pre-miRNA) that harbors the mature sequence. The pri-miRNA is cleaved in the nucleus at the base of the stem-loop structure by the RNAse III enzyme Drosha (344) and its partner DGCR8/Pasha (139, 216, 229, 330) **(FIGURE 1)**, which have been shown to require an intact secondary structure of the pri-miRNA for efficient processing (230). In this reaction, DGCR8 identifies the exact cleavage site in the 5′ and 3′ overhangs flanking the stem-loop, while Drosha catalyzes their excisions. Alternatively, a few intronic pri-miRNAs (mirtrons) bypass Drosha/DGCR8 and are processed by the splicing machinery (34, 516). In addition, miRNAs that are derived from small nucleolar RNA (snoRNA) in various species (163, 260, 528, 592) are processed by Dicer, but do not require Drosha (260). At this stage, similar to mRNA, pri-miRNAs are subject to posttranscriptional regulation. This was initially predicted due to the lack of correlation between the levels of mature miRNAs and their primary transcripts in various normal and cancer cells (610). In agreement, it was found that lin-28 binds the loop structure of let-7 and inhibits its processing by both Drosha in the nucleus (448, 638) and Dicer in the cytoplasm (518). Likewise, hnRNP A1 binds to miR-18a's loop region and regulates its processing by Drosha (426). In general, little conservation was previously observed in the terminal loop sequences of miRNAs, and it was thus believed that it does not have any functional relevance. However, upon closer examination, Michlewski et al. (426) noted that 14% of pre-miRNAs have highly conserved terminal loops. This suggested that the loop sequence in these miRNAs might play a role in their processing. In support, targeting the loop sequences by antisense oligos inhibited the processing of miRNAs that harbored conserved (miR-18a, miR-101, let-7, miR-379, miR-31), but not nonconserved (mmiR-16–1 and miR-27a), loop sequences. Following completion of this nuclear processing step, the stem-loop pre-miRNA is exported out of the nucleus via exportin-5 in a RanGTP-dependent fashion (50, 394, 692) and into the cytoplasm, where it is further processed by Dicer to yield the functional single-stranded mature 20–22 ribonucleotide construct (259) **(FIGURE 1)**.

## C. Mechanisms of Function of miRNAs

miRNAs are mainly negative regulators of mRNA translation and exert their effects through various mechanisms **(FIGURE 1)**. As a first step, the pre-miRNAs are assembled in large protein complexes known as miRNA RNA-inducing

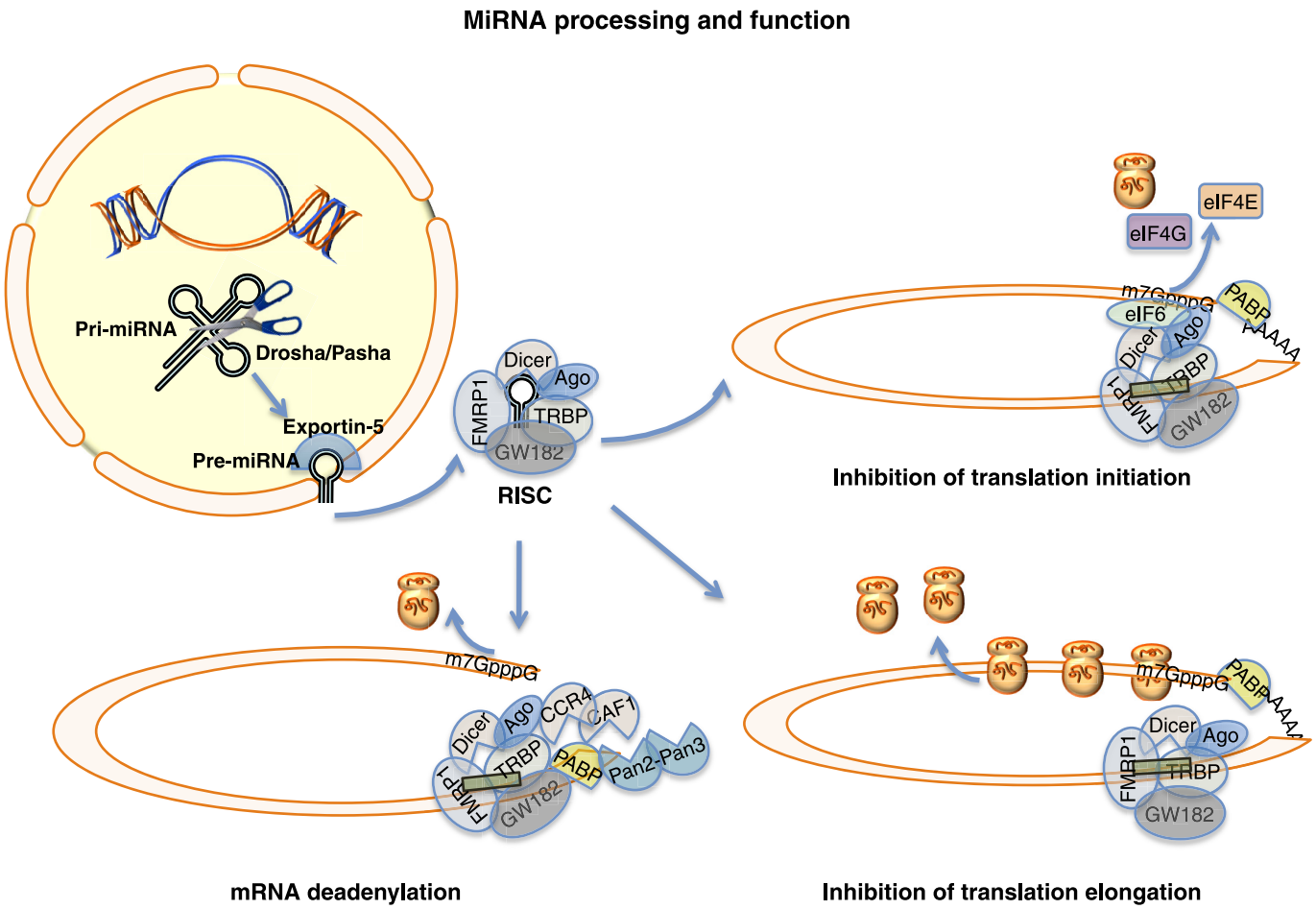


**FIGURE 1** An illustration of the steps and molecules involved in the processing and function of miRNAs. The first processing step occurs in the nucleus, where pri-miRNA is cleaved via the enzymes Drosha and Pasha. The pre-miRNA hairpin structure is then exported to the cytoplasm via exportin-5. In the cytoplasm it assembles in RISC which includes Dicer, argonaute (Ago) protein, PW182 a P-body protein, the human immunodeficiency virus transactivating response RNA-binding protein (TRBP), and fragile X mental retardation protein (FMRP1). In RISC, the loop of the pre-miRNAs is cleaved and the mature strand is used to direct the complex to its target site in the 3′-UTR of an mRNA. RISC is involved in 3 different functions: inhibition of translation initiation, inhibition of translation elongation, or mRNA deadenylation, which would result in mRNA degradation. These functions require additional recruitment of eukaryotic initiation factor 6 (eIF6). Inhibition of translation initiation is a result of disengagement of the 80S ribosomal complex in a cap (m7GpppG)-dependent manner that is partly attributed to the recruitment of the anti-association factor eIF6 by the RISC complex, or competition of Ago2 with eukaryotic initiation factor 4E (eIF4E) or eIF4G. Deadenylation occurs via interaction of Ago2 with chromatin assembly factor 1 (CAF1) and, thus, recruitment of the CCR4-NOT protein complex that harbors both chemokine (C-C motif) receptor (CCR4) and CAF1 deadenylase enzymes, in addition to a second deadenylation step involving the PABP-dependent poly(A) nuclease 2 (Pan2)-Pan3 deadenylase complex.

silencing complex (RISC or miRISC). Some of the known proteins that form RISC include the RNA-specific endonuclease Dicer, which is involved in the processing of pre-miRNA into the mature form (259); the argonaute (Ago) protein, which has four isoforms, of which only Ago2, also known as "slicer," has the capacity to cleave the target mRNA (414, 455); PW182 a P-body protein (376); the human immunodeficiency virus transactivating response RNA-binding protein (TRBP), which recruits Ago2 to the complex (100); and fragile X mental retardation protein (FMRP1) (277), which is known to associate with polyribosomes (177); among other proteins whose functions within the RISC have not been fully examined yet (246). Guided by partial sequence complementarity between the miRNA and its target, the complex binds to miRNAs target sites, mainly within the 3′-UTR of genes. It was first noted in *C. elegans* that miRNAs have the capacity to inhibit translation of its target mRNA at a postinitiation step, as lin-4 inhibits the translation of lin-14 without reducing mRNA abundance, polyadenylation, or polyribosomal content (461). Similar results were observed with inhibition of lin-41 by let-7 in a HeLa cell background, where the suppressed mRNA was associated with active polyribosomes comparable to its unsuppressed counterpart (454). In

support of this mechanism, cap-independent translation of mRNAs is also successfully inhibited by recombinant miRNA (483). It was suggested that this effect could be a result of premature dissociation of ribosomes during translation elongation.

A second mode of function employed by miRNAs is inhibition of translation initiation, which requires both the cap (253, 487, 608) and poly(A) tail structures (253). It has been known that both of these structures act in concert to facilitate translation. This is demonstrated by a model in which eukaryotic initiation factors 4G (eIF4G), a subunit of the 5′cap-binding initiation factor, binds the poly(A)-binding protein (PABP) and promotes circularization of the mRNA molecule, a structure that is translationally superior to the linear form (521). A cell-free translation system shows that miRNA inhibits translation via inhibiting the assembly of the 80S ribosomal complex in a cap (m7GpppG)-dependent manner (406). This effect could be partly attributed to the recruitment of the anti-association factor eIF6 by RISC, which prevents the assembly of the 80S complex (99). Additionally, the Ago2 protein within the RISC complex contains a cap-binding domain that competes with eIF4E and inhibits binding of the initiation complex to the m7GpppG cap (302). Ago2 also has the capacity to compete with eIF4E for its interaction with eIF4G and further disrupts assembly of the initiation complex (268).

A more elaborate mechanism became evident when it was discovered that miRNAs also induce deadenylation of mRNAs (27, 641, 671), which succeeds translational inhibition but is independent of it (168). This function is mediated through the interaction of Ago protein with chromatin assembly factor 1 (CAF1), which recruits the CCR4-NOT protein complex, harboring both chemokine (C-C motif) receptor (CCR4) and CAF1 deadenylase enzymes (168). This, in addition to GW182's interaction with PABP, induces mRNA deadenylation, which may be associated with an additional step involving the PABP-dependent poly(A) nuclease 2 (Pan2)-Pan3 deadenylase complex (88). However, the results of the latter study argue against direct physical association between miRISC and any of the deadenylase enzymes and suggests an alternative model in which miRNAs renders mRNAs more liable to deadenylation via the degradation machinery, plausibly, through disrupting PABP's association with the poly(A) tail (88). In general, deadenylation leads to destabilization of mRNA. While mammalian miRNAs are commonly recognized for inhibiting translation versus inducing mRNA degradation, there is substantial evidence that supports the latter (22). Indeed, Farh et al. (170) noted that the levels of tissue-enriched miRNAs inversely correlated with levels of some of their mRNA targets compared with their concentrations in other tissues. The question of whether a miRNA induces translation inhibition versus mRNA degradation is best addressed at the level of individual miRNAs, specific targets, and cell backgrounds.

Remarkably, miRNAs have also been observed to enhance translation in quiescent cells (631). Specifically, miR-369–3p enhances the translation of TNFα in serum-deprived, cell cycle-arrested, 293HEK cells. Interestingly, this effect switches to an inhibitory one, upon reentry of the cells into the cell cycle. A similar effect was seen with the regulation of high mobility group A2 (HMGA2) protein and its targeting miRNA, Let-7, suggesting the ubiquity of this mechanism, but not with siRNA. During miRNA-enhanced translation, it was noted that the FMRP1 protein is recruited to RISC; however, the full mechanism of this functional aspect of miRNAs awaits elucidation.

## D. Identifying the mRNA Targets of miRNAs

To appreciate the significance of a change in the level of a certain miRNA during health or disease, it is essential that we identify its mRNA targets. For that purpose, several computational target prediction tools have been developed that apply various distinct as well as overlapping algorithms, which continue to be modified as more targets are being experimentally validated. In this section, we briefly highlight some of the prominent prediction algorithms that are used by the three most widely utilized engines that generally rely on base pairing between the "seed sequence" of the miRNA and the 3′-UTR of its target, in addition to evolutionary conservation of the targeted sequence. It should be noted that while "most functional mRNA-miRNA pairing resides in the 3′-UTRs," miRNA target sites are also found within the coding region of a gene, albeit, at a much lower frequency (352). The miRanda algorithms include the following (36, 279): *1*) an alignment score between a miRNA and its target that is "the sum of match and mismatch scores" for base-pairing and gap penalties. *2*) The alignment score involves an asymmetric Watson-Crick (W-C) 5′ to 3′ base-pairing, with more weight assigned to the 5′ end (seed sequence), which can, to some degree, be compensated for by stronger complementation between the 3′ end of the miRNA and its target, while allowing mismatches and G:U wobbles in the seed sequence. *3*) Determining the degree of evolutionary conservation of the targeted sequence and its position in 3′-UTRs of human, mouse, and rat genes. TargetScan prediction software applies the following parameters (186, 220, 353): *1*) an alignment score for W-C base-pairing of nucleotides 2–7 (seed sequence) of the miRNA to its target, in addition to base-pairings beyond the seed sequence that can compensate for mismatches in the seed; *2*) it also recognizes a conserved adenosine at the first position and/or a W-C match at position 8; *3*) context scoring, which evaluates the AU content 30 nt flanking both sides of the targeted site, in addition to the latter's distance from the end of the 3′-UTR; and *4*) determining the degree of conservation using three different

levels: highly conserved between human, rat, mouse, dog, and chicken; conserved between human, mouse, rat, and dog; and poorly conserved among any combination of species. A third target prediction software, PicTar, searches for (170) *1*) a perfect W-C base-paired ~7 nucleotide seed at position 1–7 or 2–8; however, mismatches are allowed if the free binding energy does not increase. *2*) It takes into account the binding energy of the entire miRNA-mRNA duplex and *3*) the degree of conservation of targeted sites between all the species listed above in addition to chimpanzee, pufferfish, and zebrafish (316). One other important criterion that should be considered during miRNA target prediction is the accessibility of the targeted site (296, 381). It was experimentally proven that target sites that are embedded in a closed stem structure do not allow effective inhibition by the targeting miRNA (296). For that purpose, Kertesz et al. (296) designed the prediction software PITA (Probability of Interaction by Target Accessibility), which allows prediction of target sites by first searching for complementary "seed" sequences, followed by calculations of the free binding-energy after substracting the energy used to unwind any predicted secondary structures (296). Likewise, Long et al. using Sfold (144) describe a two-step model for miRNA-target binding, in which the miRNA first hybridizes to a few accessible nucleotides (nucleation), followed by its expansion as it disrupts any secondary structures (381).

It should be noted that nonconserved miRNAs-targeted sites respond equally well to inhibition by miRNAs (170). However, these nonconserved sites appear to be present in genes that are not expressed in the same tissue as the targeting miRNAs. On the other hand, genes that preferentially coexist with miRNAs in a specific tissue are thought to have evolved through selective elimination of the targeting sites and are known as "antitargets" (170). Hence, there are also target prediction applications that do not rely on target site conservation, such as MicroTar, which may be useful in some instances (606). Note that while the three target prediction applications described above overlap in many predicted targets, they diverge in others. Thus it might be beneficial to search all the databases for potential targets of a miRNA of interest. However, one needs to take into account that not all predicted targets are genuine, as they may be subject to spatial or temporal restrictions. In addition, binding to the targeting site might be modulated by 3′-UTR *cis*-acting sequences or transacting factors. Moreover, a single 3′-UTR may be targeted by multiple miRNA. Thus the level of a mRNA or its translation product is governed by the combinatorial effect of its targeting miRNA.

While computational analysis predicted the structural basis for miRNA:target pairing, Brennecke et al. (61) experimentally validated some of the parameters described above. Essentially, they confirmed that nucleotides 2–8 from the 5′-end of a miRNA are the most critical in establishing base pairing with the target. This criterion could be relaxed to 2–5 nucleotides, under conditions where the nucleotides in the 3′-end had strong complementarity with the target. Also, the position of the base pairing rather than the pairing energy determined the functional efficiency of the miRNA. It is also worth noting that a miRNA's targeting capacity might be modified by selective posttranscriptional editing of its precursor. This process converts adenosine to inosine, catalyzed by adenine deaminase in double-stranded RNA substrates (reviewed in Ref. 398). Luciano et al. (392) were the first to notice that premature miR-22 is edited at multiple sites. It is further estimated that ~6% of human miRNAs are subject to posttranscriptional editing (48). Significantly, some of the edited sites are within the "seed" region, which would accordingly lead to reassignment of targets (292).

Before proceeding with the role of miRNAs in organogenesis and pathogenesis, we should briefly describe the nomenclature system as defined in miRBase, the online repository for miRNA sequence data and target prediction (219). miRNAs are designated by a three-letter prefix, such as miR or let, followed by a number (e.g., miR-1). If a given miRNA is expressed from multiple genes, an additional corresponding numeric suffix is added, e.g., miR-1–1 and miR-1–2, while homologus miRNAs are identified by a common numeric suffix, followed by a distinct letter (e.g., Let-7a, Let-7b, Let-7c…, etc.). In some cases, two mature miRNAs are processed from the same stem-loop precursor, one from each arm, and are accordingly designated by an additional suffix, 5p for that released from the 5′-arm and 3p for that released from the 3′-arm (e.g., miR-199a-5p and miR-199a-3p).

## II. MicroRNAs IN THE VASCULAR SYSTEM

### A. miRNAs in Vasculogenesis and Angiogenesis

Early indication that miRNAs are involved in the development of the vascular system was uncovered by a generalized genomic knockout of the gene for the miRNAs processing enzyme Dicer in a mouse model. Loss of Dicer led to stunted growth and embryonic lethality that was associated with retardation in the expansion of the yolk sac's and embryo's vasculature (690). Although the exact nature of the defect was unclear, it appeared that progression, but not the initiation, of vasculogenesis was interrupted. This was confirmed by the knockdown of Dicer in human umbilical vascular endothelial cells (HUVEC), which exhibited defective proliferation, migration, and tubulogenesis (321, 584). Subsequently, mice models with targeted deletions of specific miRNAs enriched in endothelial or vascular smooth muscle cells (VSMC) were engineered to more precisely dissect their

roles in vasculogenesis and angiogenesis. miR-126 is one that is highly expressed in the heart and vasculature of zebrafish (661). It is also highly expressed in murine lung and heart and to a lesser extent in the brain, liver, and kidney, where it is localized to endothelial (235) and epithelial cells (222). Deletion of one copy of this gene results in ~50% reduction of mature miR-126 with no apparent phenotype, while deletion of both copies results in ~50% embryonic lethality due to systemic edema and widespread hemorrhage from ruptured vessels (322, 652). Additionally, vascularization of the brain at E10.5 and the retina at P0 was severely retarded. Thus the phenotype appears to involve weakened vascular walls, as well as defective vascularization in some organs. In support of the latter, endothelial cell proliferation and tubular outgrowth formation were impaired. Those embryos that escaped the lethal phenotype developed normally with no apparent abnormalities, until they were challenged with ischemia, which demanded angiogenesis. As a result, when the left coronary artery was occluded, angiogenesis was compromised in the ischemic hearts, which resulted in precipitous cardiac failure (652). This suggests that miR-126 is required for proper formation of endothelial cell-to-cell contacts and vasculatization during early development, as well as angiogenesis during ischemic conditions.

Two of miR-126's validated targets that were upregulated in the knockout models include Spred-1 (652) and the p85$\beta$ regulatory subunit of phosphatidylinositol 3-kinase (PI3K) (180, 322), although evidence for their involvement in the underlying phenotype is only circumstantial at this point. The regulatory p85 subunit is necessary for the stability and recruitment of the catalytic subunit of PI3K and, thus, the activation of the PI3K-AKT pathway (696). Indeed, knockout of p85$\alpha$-$\beta$ in endothelial results in reduced AKT activity and a phenotype reminiscent of that of the miR-126 knockout mice, including embryonic lethality due to rupturing of vessels and hemorrhaging (700). Thus the upregulation of p85$\beta$ in the miR-126 knockout models does not reconcile with the observed phenotype and requires further assessment. On the other hand, Wang et al. (652) show for the first time through gain- and loss-of-function experiments in an aortic ring sprouting assay that Spred-1 is sufficient for inhibiting vascular outgrowths (652). However, its role in vivo may be more complex, since inactivating mutations of spred-1 have been associated with neurofibromatosis 1-like phenotype in humans (60), while targeted deletion in mice induces a mild phenotype that exhibits a slightly lower body weight and shorter face (265). Also, spred-1 and spred-2 double knockout increased lymphatic vessels and lymphatic endothelial cells but had no effect on the number of blood vessels (598). Thus the miR-126 knockout phenotype might be attributed to a broader array of targets that remain to be identified.

Other miRNAs that may also contribute to vessel formation include miR-130a, which is upregulated in endothelial cells during tubulogenesis and facilitates the process by targeting and inhibiting the expression of antiangiogenic transcription factors GAX and HOXA5 (97). Similarly, miR-210, which is induced by hypoxia (72, 171), was sufficient for inducing tubulogenesis in HUVEC cells (171). However, proof of its direct targeting and suppression of EphrinA3 under these conditions does not accurately explain its function and should be cautiously regarded, since eph-ephrins are generally known to be essential for angiogenesis and neovascularization (648). Additionally, let-7f and miR-27b are also necessary for tubulogenesis, although their targets have not been investigated (321).

In contrast, overexpression of miR-92a in endothelial cells blocked tubulogenesis and was associated with reduced cell migration and adhesion, but did not affect viability or proliferation (52). Indeed, knockdown of miR-92a with antagomirs during limb and myocardial ischemia enhanced angiogenesis and, thus, improved blood flow and tissue viability in the region. One of miR-92a's validated targets that may be implicated in this process is $\alpha$5-integrin, although, undoubtedly, other targets must also be involved. miR-92a is a member of the miR-17~92 cluster, which is induced by Myc in colon cancer cells (140). Overexpression of this cluster with Ras in p53-null colonocytes did not influence cell proliferation, however, similar to Myc, when implanted in mice it enhanced tumor growth by increasing neovascularization (140). These results may be in part attributed to targeting and inhibiting thrombospondin and CTGF by miR-19 and miR-18, respectively. This reconciles with the fact that c-*myc* induces an angiogenic switch by posttranscriptionally downregulating thrombospondin-1 (270, 450, 613) and provides an explanatory mechanism for it. Thus, although overexpression of miR-92a inhibits angiogenesis, overexpression of the whole cluster enhances angiogenesis in tumors. This suggests that the function of individual members of the cluster does not necessarily reflect the function of the group.

While miR-126 is preferentially expressed in endothelial and epithelial cells, the miR-145/miR-143 cluster is enriched in vascular and visceral smooth muscle cells as early as E11.5 (678) and throughout adulthood (49, 160, 678), in addition to the embryonic heart (49, 678). Genomic deletion of this cluster resulted in viable mice with a mild vascular phenotype but no cardiac abnormalities (49, 160, 678). In particular, the smooth muscle cells were smaller and exhibited an increase in rough endoplasmic reticulum and a decrease in actin stress fibers, with a resultant thinner tunica media. In addition, there were small neointimal lesions and a megacolon phenotype, which suggested that knockout of miR-145/143 may inhibit differentiation and, subsequently, enhance proliferation and migration of smooth muscle cells (160). However, the failure to detect a

consistent increase in VSMC proliferation or apoptosis argued that the miRNA may not be directly required for differentiation but rather for the ability of VSMC to differentiate in response to the physiological contractile demand (49). In concordance, this phenotype was accompanied by lower blood pressure (49, 678) and depressed responsiveness to vasopressors (49), in spite of normal levels of smooth muscle $\alpha$-actin and myosin heavy chain, although studies differed on this latter finding (160). On the other hand, the expression of caldesmon, calponon, and smoothelin was reduced in the miR-145/143-devoid VSMC (49). A separate study showed that miR-145 is also enriched in a subset of pericytes in adult brain and kidney microvessels and inhibits cell migration, plausibly by targeting Friend leukemia virus integration I (Fli 1) (333). Fli 1 belongs to the ETS-1 family of transcription factors that play a critical role in angiogenesis (486). Several other downstream targets have been identified and validated that could potentially mediate miR-145/143 effects; these include kruppel-like factor 4 and 5, adducin-3, slit-robo GAP-1 and -2, slingshot 2 phosphatase, and MRTF-B, which are thought to play a role in cytoskeletal remodeling in VSMC (678). However, the functional dynamics between these molecules and how they specifically contribute to the miR-145/143 knockout phenotype remain to be examined. On the other hand, angiotensin converting enzyme (ACE), which has been validated as a miR-145 target that is derepressed in the knockout model, may be the cause of desensitization and downregulation of angiotensin I receptor, thus explaining the lower blood pressure and irresponsiveness to vasopressors (49). In support, treatment of the mice with an ACE inhibitor partially alleviated these signs, but was not sufficient for reversal of other aspects of the phenotype.

Although the results of genomic ablation of miR-145/143 in mice would argue against its involvement in cell specification or fate, cell culture studies demonstrate that increased expression of miR-145 in human embryonic stem cells is both necessary and sufficient for inducing their differentiation into mesoderm and ectoderm lineages through suppression of OCT4 and SOX2 (680). Likewise, knockdown of miR-145 in myocardin-treated fibroblast resulted in inhibition of their transformation into VSMC, while its overexpression in neural crest stem cells was sufficient for inducing their commitment to the VSMC lineage and expression of smooth muscle actin and myosin heavy chain (117). The discrepancy between the knockout models and the cell culture experiments is readily explained by the impact of a cell's environment on its response to stimuli.

### B. miRNAs in Neointimal Hyperplasia

Dedifferentiation of VSMC is an underlying mechanism in neointimal hyperplasia and, thus, vascular stenosis during atherosclerosis or vascular injury. Given the role of miR-145 in VSMC that is described above, it is not surprising to learn that this miRNA is downregulated in response to vascular injury (103). While the knockout models dispute the fact that downregulation of miR-145 is sufficient for reducing $\alpha$-actin and myosin heavy chain (49, 678), they do show that abrogation of miR-145 induces limited neointimal lesion formation (49, 160). In particular, one study has shown that these lesions are only observed in the femoral artery of older knockout mice (18 mo), which suggested that although downregulation of miR-145/143 does not result in fulminant neointimal formation, it does promote it (49). In consensus, restoring miR-145 levels during vascular injury inhibits neointimal formation (160, 678), with promising therapeutic prospects. miR-145's effect in this context could be, at least partially, explained by the suppression of its target, Krupple-like 5 (Klf5). Indeed, KLF5, first identified as a transcription factor that induces embryonic smooth muscle myosin heavy chain (656), is involved in enhancing smooth muscle proliferation by upregulating cyclin D1 (588).

In contrast to miR-145, miR-221 (133, 378), but not the co-clustered miR-222 (133), positively regulates smooth muscle proliferation. It is induced by platelet-derived growth factor (PDGF), which is known to stimulate VSMC switching and proliferation during angiogenesis and neointimal formation (133, 378). Gain- and loss-of-function experiments proved that it is indeed required for mediating the effects of PDGF via targeting and suppressing the cell cycle inhibitors $p27^{Kip1}$ (133, 378) and $p57^{Kip2}$ (378), as well as c-*kit* (133). In concordance, knockdown of miR-221 during vascular injury reduced neointimal thickness by 40% (378). The effects of miR-221 are likely to be reinforced by the concurrent upregulation of miR-21 in neointimal lesions (275). miR-21, which is a ubiquitously expressed prosurvival miRNA, was shown to inhibit PTEN expression under these conditions (275, 416, 417). Accordingly, its abrogation increased apoptotic VSMC death and reduced neointimal thickness. Thus neointimal formation is the result of a combinatorial effect of changes in the expression of several miRNAs and their targets that regulate VSMC differentiation, proliferation, and survival.

## III. MicroRNAs IN THE HEMATOLOGICAL SYSTEM

### A. miRNAs in Lymphopoiesis, Lymphocytes, and Adaptive Immunity

A role for miRNA in B-cell development was demonstrated by targeted deletion of Dicer, which resulted in the arrest of pro-B to pre-B cell transition (312). In particular, some of the mature miRNAs that were depleted included miR-17, miR-142–3p, miR-181, and miR-191. The phenotype could partly be attributed to pre B cell apoptosis as a consequence of the increase in the proapoptotic protein Bim, a

miR-17~92 target **(FIGURE 2)**. Knockout of miR-17~92 confirmed that this phenotype is indeed predominantly attributed to this miRNA cluster versus any of the others that were depleted by the loss of *Dicer* (633). Aside from the arrest of B-cell development, the knockout had no effect on erythropoiesis or myelopoiesis; however, the homozygous mice died at birth due to lung and cardiac ventricular septal defects. In contrast, overexpression of miR-17~92 in lymphocytes resulted in an increase in peripheral lymphocytes, especially the CD4+ T cells, which in older mice expressed the activation marker CD69 (676). In addition, there was an increase in B1 cells and the germinal centers in lymph nodes and spleen, which was associated with an enlarged spleen and lymph nodes; an increase in circulating IgG2b, IgG2a, and IgG3; infiltration of organs by lymphocytes; and deposition of immune complexes, suggestive of an autoimmune reaction in these mice. Thus miR-17~92 appears to impact B-cell number by enhancing their viability, which may be partly attributed to suppression of Bim.

Activation of CD4+ T lymphocytes results in rapid and sustained upregulation of the noncoding BIC transcript (227), whose function was ambiguous until it was discovered that it harbors miR-155 (155, 707). Similar to its host gene, miR-155 is initially very low in hematopoietic stem cells (HSC) and mature hematopoietic cells. The function of this gene was eventually uncovered by targeted deletion in a mouse model. Except for thickening of the bronchial walls (510) and reduced B cells in the germinal centers of Peyer's patches and mesenteric lymph nodes (607), loss of miR-155 had no impact on general development, including that of the myeloid and lymphoid systems (510, 607). However, the B cells' immune response was impaired in these mice, as they produced less IgM and exhibited reduced antigen-specific antibody switch. In addition, T-cell function was also impacted, producing less interleukin (IL)-2 and interferon (IFN)-$\gamma$ partly due to defective antigen presentation by dendritic cells. Although miR-155 knockout did not affect differentiation of T helper (Th) cells, it did result in Th1 cells

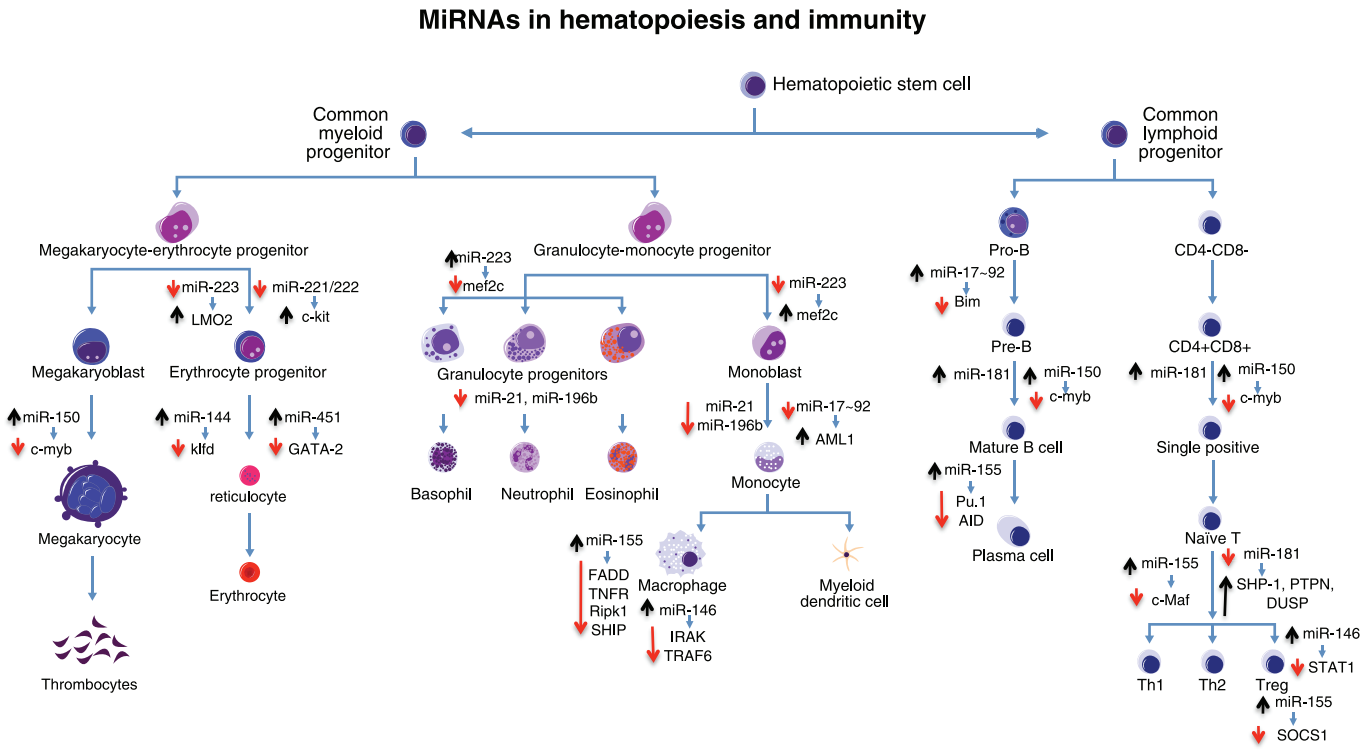


**FIGURE 2** A diagram showing miRNAs and their targets that play a role in the development of the hematopoietic system. The diagram lists the various miRNAs and their targets that are involved in hematopoiesis. Upregulation or downregulation of a specific miRNA is represented by an upward (black) or a downward (red) arrow, respectively. The changes in the expression levels of a target gene inversely correlate with that of the targeting miRNA and are similarly represented by an up or down arrow. All listed targets have been validated. The miRNA targets listed include LIM domain only 2 (LMO2), v-*kit* Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (c-*kit*), v-*myb* myeloblastosis viral oncogene homolog (c-*myb*), Kruppel-like factor d (klfd), GATA binding protein 2 (GATA-2), myocyte enhancer factor 2C (mef2c), acute myeloid leukemia 1 protein (AML1), Fas (TNFRSF6)-associated via death domain (FADD), tumor necrosis factor receptor (TNFR), receptor (TNFRSF)-interacting serine-threonine kinase 1 (Ripk1), SH2 containing inositol phosphatase, isoform b (SHIP), IL-1 receptor-associated kinase 1 (IRAK), TNF-receptor-associated factor 6 (TRAF6), bcl-2 interacting mediator of cell death (Bim), hematopoietic transcription factor PU.1 (Pu.1), activation-induced cytidine deaminase (AID), v-maf musculoaponeurotic fibrosarcoma oncogene homolog (c-Maf), protein-tyrosine phosphatase (SHP-1), non-receptor type tyrosine phosphatase (PTPN), and dual specificity tyrosine phosphatase (DUSP).

that produced higher levels of chemokine (C-C motif) ligand 5 (CCL5) and an increase in Th2 cells, along with elevated levels of IL-4, IL-5, and IL-10, possibly a result of an increase in c-Maf, a miR-155 target. In contrast, regulatory T cells (T-reg) numbers, but not function, were reduced in the knockout mice (309). The defective response of B cells in this model could be attributed to an increase in PU.1, which reduces IgG1 switching and is a target of miR-155 (636). On the other hand, miR-155 has also been reported to target the activation-induced cytidine deaminase (AID) gene (149, 604). In these studies, mutation of the miR-155 target site within the 3′-UTR of the endogenous AID gene resulted in higher levels of the protein in B lymphocytes undergoing class switch recombination. At a first glance, these results might appear to contradict the decrease in class switching observed in the miR-155 knockout models; however, concurrent upregulation of PU.1 might counterbalance the effects of AID. This emphasizes the need not only to identify all targets for a given miRNA in a temporal and spatial manner, but also to characterize their functional interaction.

miR-155 is regulated by Foxp3 and was indeed confirmed necessary for the optimal proliferation of thymic and peripheral Foxp3+ T-reg cells (389). This function is a consequence of its direct targeting of suppressor of cytokine signaling 1 (SOCS1), which results in enhanced IL-2-induced activation of signal transducer and activator of transcription 5 (STAT5). On the other hand, T-reg-specific Dicer knockout proved that the suppressor activity of T-reg also requires miRNAs, as these mice exhibited a lethal autoimmune response when challenged with an inflammatory condition (374). A recent report shows that, in this case, miR-146a is the miRNA responsible for T-reg cells' suppressor function (388). In contrast to miR-155 knockout, miR-146a mice had more T-reg cells, but their suppressor function was impaired. Accordingly, a miR-146a$^{-/-}$/Foxop3KO mouse exhibited an autoimmune lethal phenotype similar to that observed in the T-reg-specific Dicer knockout mouse. The underlying defect appears to be resultant from an increase in miR-146a's target STAT1, which enhances the production of IFN-$\gamma$ and, thereby, exaggerates Th1 development and autoimmunity.

In contrast to miR-155, miR-150 is highly expressed in mature B and T lymphocytes **(FIGURE 2)**. When prematurely expressed, though, it halts B-lymphocyte maturation at the pro-B cell stage, while its effect on T-cell development was inconsistent (675, 714). In contrast, miR-150 knockout mice developed normally, including a typical T-cell, follicular and marginal zone B-cell populations, except for an increase in peritoneal and splenic B1a cells, known for recognizing self-antigens (675). As a direct target of miR-150, c-*myb* is upregulated in this model. The phenotype of the transgenic and knockout models is consistent with the phenotype previously observed in B cell-targeted knockout of c-myb, in which B-cell development was impaired at the pro- to pre-B conversion step with loss of the B1 subtype (609).

Similar to miR-150, miR-181 is relatively low in HSC, then specifically increases in differentiated B lymphocytes (89). In this case, when ectopically expressed in HSC, it results in ~2.5-fold increase in B lymphocytes versus an 88% decrease in T lymphocytes (89). miR-181 is also enriched in the thymus, where it is high during the early stages of T-cell differentiation and gradually declines to its lowest level in Th1 and Th2 cells (359). Its overexpression in antigen primed T cells not only increases their sensitivity to peptide antigens but also suppresses T-cell receptor antagonism. This effect is likely accomplished through downregulating multiple phosphatases that have a negative-feedback effect on T-cell receptor (TCR) stimulation. Those identified as miR-181 targets include protein tyrosine phosphate SHP-1, non-receptor type tyrosine phosphatases (PTPN), and dual-specificity phosphatases (DUSPs). The data suggest that the higher levels of miR-181 in the immature double-positive T cells versus the mature cells may be involved in heightening their sensitivity to low-affinity self-antigens and thus may play a role in establishing self-tolerance. Additionally, a recent study shows that miR-181 is necessary for negative selection of self-reactive thymocytes (153). Thus miR-17~92, miR-155, miR-150, and miR-181 play a role in fine-tuning the adaptive immune response. However, while their functions have been individually examined, their combinatorial effect remains to be elucidated.

## B. miRNA in Myelopoiesis, Granulocytes/Monocytes, and Innate Immunity

One of the bone marrow-enriched miRNAs includes miR-223, which is most abundant in the myeloid cell lineage (89). Its expression continues to increase as the cells develop into granulocytes, but conversely, it declines if they differentiate into monocytes (281) **(FIGURE 2)**. Consistent with its developmental pattern, this gene is regulated by c/EBP (173, 190) and PU.1 (190) transcription factors, which is characteristic of myeloid-specific genes. Counter to predictions, though, knockout of miR-223 did not disrupt the development of leukocytes or monocytes, but did enhance proliferation of the granulocyte-monocyte progenitors, as well as their differentiation into granulocytes, resulting in neutrophilia (281). This effect was reversed by knockout of Mef2c that serves as a target of miR-223 and, thus, a mediator of its effects. In addition to an increase in their number, the miR-223-deficient neutrophils proved to be hyper-reactive and more efficient in eliminating target organisms. Accordingly, when challenged in vivo with sublethal doses of lipopolysaccharide (LPS), the mice exhibited a hyperinflammatory response that led to liver, spleen, and muscle necrosis, but unlike the neutrophilia, this effect was not mediated by Mef2c, suggesting that other miR-223 targets are in play.

This represents another example of miRNAs' regulation of precise developmental timing of progenitor cells.

While miR-223 is preferentially involved in differentiation of granulocytes, miR-17~92 cluster and its paralog miR-106~25 are involved in the differentiation of monocytes (181). During differentiation of unilineage monocytic cultures, downregulation of these miRNA clusters is necessary for the upregulation of the transcription factor acute myeloid leukemia 1 (AML1) and its transcriptional target monocyte-colony stimulating factor receptor (M-CSFR). Reciprocally, AML1 functions as a transcriptional inhibitor of these miRNA clusters, creating a double negative-feedback loop. On the other hand, growth factor independent 1 (Gfi1) transcription factor is required for the differentiation of both granulocytes and monocytes (287) and appears to function through suppression of miR-21 and miR-196b whose targets in this cell type remain to be identified (632).

As miRNAs are involved in the development of the myeloid cell lineage, they, in turn, impact innate immunity. miR-155 is one of the most substantially upregulated miRNA after stimulation of macrophages with IFN-$\beta$ (458) or TNF-$\alpha$ (614). Consequently, miR-155 targets and suppresses I$\kappa$B kinase epsilon (IKK$\epsilon$), Fas-associated death domain protein (FADD), and receptor (TNFR superfamily)-interacting serine threonine kinase 1 (Ripk1), which are known to be involved in LPS and TNF-$\alpha$ signaling. Likewise, miR-155 is upregulated in the bone marrow in nonlymphocytic cells following LPS administration (457). Direct evidence of the involvement of miR-155 in innate immunity is provided by the fact that ectopic expression of miR-155 in HSC via retroviral delivery induces an increase in granulocytes/monocyte (GM) population and a reduction in erythroid and B-cell precursors and megakaryocytes in the bone marrow, which correlates with higher GM in the circulation, anemia, and thrombocytopenia (457). On the other hand, the spleen enlarges due to compensatory extramedullary hematopoiesis. This phenotype has been attributed to the direct suppression of the Src homology-2 domian containing inositol-5 phosphatase (SHIP) gene, a negative regulator of the AKT pathway that proved to be a direct target of miR-155 (121, 456). When knocked down with short interfering RNA, this gene induces a very similar phenotype to that observed with the overexpression of miR-155 (456). However, it partially overlaps with that of the genomic knockout of SHIP, which only exhibits reduced B cell precursors in the bone marrow (243). Overall, these results suggest that miR-155 is involved in the restoration of granulocytes and monocytes levels after their depletion by an infection. These effects appear to be counterbalanced by miR-146, which is also induced by proinflammatory stimuli in macrophages (593). This miRNA contributes to a negative-feedback effect through direct suppression of the translation of IL-1 receptor associated kinase 1 (IRAK1) and TNF receptor-associated factor 6 (TRAF6).

### C. miRNAs in Thrombopoiesis

miR-150 is also more abundant in human megakaryocytes relative to their megakaryocyte-erythrocyte progenitor (MEP) or erythrocytes (385) **(FIGURE 2)**. Indeed, overexpression of miR-150 shifts differentiation of MEP towards megakaryocytes at the expense of erythrocytes both in cultured MEP and in vivo. This effect is mediated by its target myb, which proves to be consistent with previous studies showing that mice expressing an inactive mutant of c-*myb* exhibited an increase in megakaryocytes and reduced erythrocyte progenitors (422). In contrast to miR-150, overexpression of miR-155 in HSC reduced the number of megakaryocytes in the bone marrow (457), plausibly due to its suppression of the transcription factors Ets-1 and Meis1 that are involved in megakaryocyte development (512).

### D. miRNAs in Erythropoiesis

Stem cell factor (SCF) and its receptor c-*kit* play a critical role in the survival and proliferation of erythroid progenitor cells (452). It was noted that miR-221/222's expression gradually declines during the development of CD34+ erythroid progenitor cells, which inversely correlated with the expression of its predicted target c-*kit* (175) **(FIGURE 2)**. Further experiments confirmed that c-*kit* is indeed a direct target of miR-221, as ectopic expression of the miRNA depressed expression of its target, inhibited proliferation, and accelerated differentiation of erythrocyte precursors both in vitro and in vivo. Similarly, upregulation of the Lim-only protein 2 (LMO2), also required for erythroid differentiation, is a consequence of a decline in its targeting miRNA, miR-223 (650).

In contrast to miR-221/222 and miR-223, miR-451 is dramatically and specifically upregulated during the differentiation of erythroid progenitors, which suggests that it plays a positive role during that transition (405). Indeed, knockdown of miR-451 in zebrafish resulted in anemia due to defective differentiation of the progenitor cells (148, 475). Furthermore, the expression of miR-451 is driven by a GATA-1-dependent promoter (148), which reconciles with the fact that GATA-1 is also required for the differentiation of erythroid cells (189). miR-451 was also shown to be necessary, but not sufficient, for mediating GATA-1's function. Some of its targets that were proven to mediate its effect include GATA-2 (475) and c-*myc*, which is known to inhibit erythroid differentiation (148). Meanwhile, as miR-144 matures from the same transcript as miR-451, it targets and inhibits the $\alpha$-globin promoter activator krupple-like factor and induces the

developmental switch from the fetal α-hemoglobin to the adult β-hemoglobin isoform (187).

## E. miRNAs in Hematological Disorders

### 1. Lymphoma

Before the discovery of miRNA in mammalian cells, Tam et al. (596) reported that the "Bic" integration site for the avian leukosis virus, which induces B-cell lymphomas, generates a noncoding RNA. Provirus insertion into the BIC promoter increases the expression of a chimeric RNA containing Bic exon 2; however, since this transcript lacked a significant open reading frame, its role in lymphomas remained undefined until recently. After the emergence of miRNAs, it was soon discovered that this noncoding transcript harbored miR-155 (155, 707), thus potentially explaining Bic's function. The significance of the amplification of miR-155 in lymphomas was examined by ectopically expressing it in B cells in a transgenic mouse model. This resulted in pre-B-cell expansion and bone marrow replacement, splenomegaly, and lymphopenia that preceded the development of lymphoblastic leukemia and lymphoma (122). It was noted that Bic/miR-155 transcript is upregulated in select forms of lymphomas, including Hodgkin's, diffuse large B-cell (DLBCL), and primary mediastinal B-cell lymphomas (155, 306), but not primary Burkitt lymphoma (305). Interestingly, ectopic expression of Bic in a Burkitt lymphoma-derived cell line failed to increase mature miR-155 levels due to a possible block to its posttranscriptional processing (307), which reconciles with the lack of correlation between the levels of Bic and miR-155 in various lymphoma cells line (155). Although it is now well established that miR-155 plays a critical role in the development of lymphomas, its upstream regulatory pathway or downstream targets that mediate its malignant effects have not been fully elucidated. A few miR-155 targets have been validated in normal B cells and macrophages as described above, but it remains uncertain whether the same targets are relevant in the lymphoma cell background. A recent study by Pedersen et al. (479), though, does show that SHIP1 is a direct target of miR-155 in lymphoma cells, similar to what is observed in macrophages (456, 121); however, the extent to which it mediates the effect of miR-155 in this context remains to be examined. Meanwhile, miR-155's upstream regulators include TNF-α, which enhances its expression in DLBCL lymphoma cells.

Calin et al. (68) have reported that more than 50% of miRNAs are located at genomic sites that are disrupted or amplified in various cancers. Consistently, 13q21-qter region, which is amplified in DLBCL (500), mantle cell (136), and follicular (446) B-cell lymphomas, also encompasses miRNAs (463). The candidate gene, *Chromosome 13 open reading frame 25* (*C13orf25*), within the 13q31 region amplified in DLBCL is preferentially upregulated in B-cell lymphoma cell lines and DLBCL patients with 13q31-q32 amplifications (463). Similar to Bic, this gene does not include a substantial open reading frame, but does harbor a cluster of seven miRNAs (miR-17∼92) (463). In agreement, this miRNA cluster was found to be increased ∼10-fold in 65% of B-cell lymphoma samples (240). As mentioned above, knockout of miR-17∼92 inhibited B-cell maturation, whereas its overexpression induced an increase in B1 cells and germinal centers. On the other hand, when overexpressed with the myc oncogene, it greatly accelerated c-*myc*-induced pre-B cell lymphoma in mice and reduced apoptosis. Validated targets of this cluster that potentially mediate its function include the proapoptotic gene Bim (312, 633) and the cell cycle inhibitor *CDKN1A*/p21$^{waf1/Cip1}$ (264). c-Myc enhances the transcription of the miR-17∼92 gene (459), but suppresses that of miR-15a/16–1, miR-34a, miR-150, miR-195, Let-7, miR-26, and miR-29, suggesting that they may be tumor suppressors in this context (86). Reciprocally, let-7a directly targets and inhibits myc, creating a double-negative feedback loop (526). In addition to the above miRNAs, independent downregulation of miR-143 or miR-145 results in enhanced B-cell proliferation by derepressing the expression of ERK5 (6).

An imbalance in the steady-state stochiometry of a specific miRNA/the number of its targeted sites is likely to perturb the translational output of those targets. Theoretically, this could be induced by fluctuations in the level of the miRNA, mRNAs, or the number of targeted sites within the mRNAs, as a result of amplifications, deletions, or mutagenesis of those sites. Most findings hitherto have focused on changes in the miRNAs' levels that directly impact its targets. On the other hand, a substantial increase or a decrease in a mRNA will imbalance the availability of a specific miRNA that would indirectly affect other targets. With this comes the realization that in some cases the fluctuations in a miRNA's level may be a compensatory response to changes in its targets' levels under the same conditions. The third possibility is that a specific miRNA recognition site within a mRNA is altered through mutagenesis. An example of this is provided by the work of Chen et al. (93), where they demonstrated that the deletion in the 3′UTR of cyclin D1 (*CCDN1*) in mantle cell lymphoma releases that gene from the negative regulatory effect of miR-16 and, thus, enhances its translation. It is now realized that this phenomenon occurs on a much broader scale in cancer through selective cleavage and polyadenylation of mRNAs that leads to elimination of their miRNA-targeted sites and, thereby, higher translational output (410).

### 2. Chronic lymphocytic leukemia

Chronic lymphocytic leukemia (CLL) is the second most common form of leukemia in adults. The chromosomal region 13q14 is frequently deleted in CLL patients and was, thus, predicted to harbor a tumor suppressor gene (146). One of the potential candidate genes in this region is

the noncoding *deleted in lymphocytic leukemia 2* (*DLEU2*) gene (379). Interestingly, this gene was subsequently found to be the host for the *miR-15a/miR-16–1* cluster, located between its second and fifth exons (67). In addition, germline mutations were discovered in the miR-15a/16–1 precursor, which reduce its expression and are also associated with its deletion (67). In support, an inactivating mutation in the same cluster was found in the New Zealand black CLL mouse model (502). Alternatively, a small number of CLL cases that did not harbor a deletion of the miR-15a/16–1 locus had a deletion in the host gene's promoter (350). Accordingly, mature miR-15a/16–1 was significantly downregulated in 68% of CLL patient samples tested. miR-15 and miR-16 are ubiquitously expressed from two genes, *miR-15a/16–1* on chromosome 13 and *miR-15b/16–2* on chromosome 3, being most abundant in normal CD5+ B lymphocytes. Consistent with their being tumor suppressors, they directly target and inhibit the antiapoptotic gene BCL2 (111), the tumor suppressor immunoglobulin superfamily member 4 (IGSF4), and cyclins E1 and D1 (93, 350), in addition to indirectly inhibiting an array of genes related to proliferation, oncogenesis, and suppression of apoptosis (66). In agreement, overexpression of miR-16 inhibits cell proliferation (373) and suppresses tumor growth after in vivo implantation (66). While the data overwhelmingly support the fact that miR-16 is downregulated in carcinogenesis, a couple of studies have reported upregulation of miR-16 in CLL that did not correlate with the 13q14 deletion status (191, 649). However, the functional significance of this upregulation was not examined. This discrepancy between studies may reflect differences in the clinical stage or nature of the samples that were examined that may exhibit differential compensatory upregulation of the homologous miR-15b/16–2 locus. Thus a detailed time course of the expression pattern of miR-16 in CLL patient samples may help unravel the reason behind these conflicting data. Whereas the miR-15/16 cluster plays a dominant role in CLL, the roles of miR-29 and miR-181 should not be overlooked, as they are both downregulated and target the oncogene *T-cell leukemia/lymphoma 1 (TCL1)* (481).

### 3. Acute myeloid leukemia

Profiling of miRNAs in cytogenetically normal acute myeloid leukemia (AML) samples that were associated with an internal tandem duplication in the fms-related tyrosine kinase 3 gene (FLT3-ITD), wild-type nucleophosmin (NPM1), or both, resulted in identification of a miRNA signature that predicted the disease's outcome (404). Among those, miR-181 inversely correlated with event-free survival. This is consistent with the fact that miR-181 is normally present at low levels in HSC but specifically increases in differentiated B lymphocytes (89). Another differential display revealed a distinct pattern of miRNAs that differentiated between AML with mutant cytoplasmic NPM1c+ vs. normal NPM1 (198). This uncovered the preferential downregulation of miR-204 and upregulation of its validated targets HOXA10 and MEIS1 in NPMc+ AML that was associated with a more favorable prognosis. Additionally, a separate miRNA profiling revealed that AML patients with higher levels of miR-191 and miR-199a had a lower event-free survival rate (201). In general, unique miRNA signatures distinguish the different AML karyotypes (145), although more studies are necessary to determine the functional relevance of these different miRNA patterns.

There is a dearth of functional studies regarding the role of specific miRNAs in AML. AML with a *t*(8;21) translocation that generates the acute myeloid leukemia 1/ETO fusion oncogene is associated with reduced levels of miR-223 that inversely correlates with the severity of the phenotype (dedifferentiation) (172). Interestingly, AML1/ETO was found to directly bind and inhibit the promoter of miR-223 through heterochromatic silencing. This is consistent with the fact that miR-223 is upregulated during granulocyte differentiation (281) and induces differentiation in myeloid leukemic cells (172). Although knockout of miR-223 was not sufficient for the induction of frank AML, it did elicit neutrophilia (281). Another miRNA that is implicated in CLL is miR-29. Similar to CLL and other forms of cancer, miR-29 is downregulated in AML. The miR-29b-1/miR-29a cluster is located in the frequently deleted 7q32 region, whereas a second miR-29b-2/miR-29c cluster is located in 1q23. Reintroducing miR-29 into leukemic cells decreased proliferation, increased apoptosis, and inhibited tumor growth in vivo (199). Three of its relevant validated direct targets include the Cxxc6 (tet1) oncogene, the antiapoptotic Mcl1, and the cyclin-dependent kinase Cdk6. Ectopic expression of miR-29 also induces upregulation of the cell cycle inhibitors $p15^{INK4b}$ and ESR1 through demethylation of their promoters via directly targeting the methyltransferases DNMT3A and DNMT3B and indirectly DNMT1 (200).

### 4. Chronic myeloid leukemia

One of the characteristics of CML is a translocation of *t*(9;22) (640), which results in the production of the BCR-ABL fusion gene (25, 29, 135, 242). Similar to other cancer-related chromosomal aberrations described above, ~30% of Philadelphia-positive patients harboring this translocation also encompass a deletion that includes miR-199b and miR-219–2 (87). However, due to limited sample size ($n = 2$) in that particular study, neither the expression pattern nor disease correlation could be established for these miRNAs. The function of BCR-ABl involves the induction of myc, which augments it transformation capacity (531). As indicated above, myc also induces miR-17~92 expression, which enhances the development of myc-induced pre-B cell lymophoma (240). Venturini et al. (634) extended these findings to show that a BCR-ABL-myc-dependent pathway also regulates miR-17~92 in CML CD34+ cells, in which it is similarly involved in

antiapoptotic and proliferative functions. While BCR-ABL regulates the expression of miR-17~92, it is itself a target of miR-203. miR-203 is located within a fragile region on chromosome 12, where its promoter is frequently hypermethylated in Philadelphia-positive tumors versus those that are free of ABL1 aberrations (62). miR-203 directly targets and inhibits the expression of ABL1 and BCR-ABL. It is plausible that downregulation of miR-29, which is implicated in aberrant hypermethylation in CLL (200), may be involved in silencing of the miR-203 promoter in CML.

#### 5. Acute lymphocytic leukemia

Not surprisingly, ALL and CLL have distinct miRNA expression patterns (702). Moreover, ALL's unique miRNA signature could accurately distinguish it from AML (423). In particular, miR-128a, miR-128b, let-7b, and miR-223 were sufficient for discriminating between the two forms of leukemia with a 97% accuracy. miR-128a and miR-128b were higher in ALL, while Let-7a and miR-233 were higher in AML. Whereas let-7 is known for its tumor suppressive function via targeting c-*myc* in lymphoma cells (526), miR-223 has been shown to increase during granulocyte differentiation (281) and is sufficient for inducing differentiation of myeloid leukemia cells when ectopically expressed (172). In addition, downregulation of both miRNAs is the trend observed in many cancers, as described in other sections of this review. Accordingly, their higher expression in AML versus ALL does not reconcile well with the better overall prognosis associated with ALL. Thus stage-specific screening of more samples is needed to resolve this discrepancy.

Similar to CLL and CML, epigenetic regulation of miRNAs play a role in the underlying pathogenesis of ALL. Specifically, the promoter of miR-124a was found to be hypermethylated and silenced in ALL samples (4). Moreover, the extent of methylation correlated with higher mortality rates and proved to be an independent marker for overall, and disease-free, survival. Functionally, overexpression of miR-124a reduced cell growth in in vitro and in vivo models, the effects of which could be ascribed to its targeting and inhibiting the expression of the cell cycle protein, cyclin-dependent protein 6 (Cdk6), and reducing phospho-Rb. Another unique mechanism that may be involved in the etiology of ALL involves miR-125b-1. This miRNA was found inserted in the rearranged immunoglobulin heavy chain gene locus in a single ALL patient, the frequency and reproducibility of which remains to be examined (572). Furthermore, a genome-wide approach using extensive sequencing has recently identified an array of novel miRNAs in ALL that await characterization (704).

### F. Virus-Regulated miRNAs in the Immune System

#### 1. Epstein-Barr Virus

As in other pathological conditions, viral infections can perturb a cell's miRNAs levels, where some may be involved in antiviral mechanisms, others can participate in regulating the virus's latency and lytic cycles. Viruses also express their own miRNAs (v-miRNA) that regulate their production in host cells (485). Unlike eukaryotic miRNAs, though, v-miRNAs are generally not conserved. It was first noted in Epstein-Barr virus (EBV) latently-infected B cells that this virus expresses unique v-miRNAs that originates from the Bam H1 fragment H open reading frame 1 (BHRF1) and the Bam H1-A region rightward transcript (BART) viral genes (485). Recently, deep sequencing of the EBV miRNome in nasopharyngeal carcinoma revealed 44 BART miRNAs (94). The results also uncovered 5′ and 3′ end v-miRNA variants, as well as nucleotide variants that resulted from posttranscriptional editing. Some of these v-miRNAs target lytic genes, suppress viral proliferation, and sustain latency. Specifically, miR-BART2 is expressed from the antisense strand of the viral DNA polymerase BALF5 lytic gene and is, thus, 100% complementary to that gene and induces degradation of its mRNA. Additionally, an EBV miRNA, v-snoRNAl$^{124pp}$, derived from a v-snoRNA precursor targets BALF5 (260).

EBV miRNAs are also found in various types of lymphomas that have been linked to the EBV virus (674). This includes the expression of miR-BHRF1–3 in a type III versus type I BL cell line, miR-BART2 in primary unmanipulated type I BLs and EBV+ primary effusion lymphomas (PELs), and both BART2 and BHRF1–3 in DLBCLs. By targeting and suppressing IFN-inducible T-cell attracting chemokine (C-X-C motif) ligand 11 (CXCL11), miR-BHRF1–3 may potentially be involved in evasion of the immune system (674). At the same time, EBV miRNAs can suppress certain cellular proteins for a survival advantage. One example is miR-BART5, which targets p53 upregulated modulator of apoptosis (PUMA) and, thereby, enhances resistance of the host cell to apoptotic reagents (108). In addition, EBV can expand its effects by utilizing cellular miRNAs. This includes miR-155, which is upregulated in latency type III EBV-positive Burkitt's lymphoma cell lines (305). Infection of human lymphocytes with EBV proved that this is indeed a direct effect of the viral infection and is mediated via a latent membrane protein 1 (LMP1)-NF$\kappa$B-dependent mechanism (202, 384). The upregulation of miR-155 seems to be responsible for an increase in EBV copy number in latently-infected cells. Likewise, the EBV LMP1 enhances the expression of miR-146a via NF$\kappa$B in Burkitt's lymphoma cells (433). Overexpression of miR-146a in Akata cells revealed that it predominantly suppresses interferon-responsive genes and is, thus, predicted to provide the virally infected cells with an advantage

against the immune system (71). Other miRNAs that are also upregulated in latency III include miR-21, miR-23a, miR-24, miR-27a, and miR-34a (70). In contrast, miR-96 and miR-128a/b were selectively reduced in lymph nodes of EBV-positive classic Hodgkin lymphoma patients (445). It is notable, though, that Godshalk et al. (209) found that 99.5% of cellular miRNAs were downregulated after initial EBV infection of human B cells that was distinct from ligand (CD40)-induced activation. Six months later, however, miR-155, miR-21, miR-17–5p, and miR-20 were highly upregulated.

### 2. Kaposi's sarcoma herpes virus

Kaposi's sarcoma herpes virus (KSHV) infection is associated with the development of malignancies, including Kaposi's sarcoma and primary effusion lymphoma. This virus expresses 12 pre-miRNAs that are clustered in the transcript of the latency kaposin gene (484, 524). Deletion of 10 of these miRNAs (K-1 through -9 and -11) results in upregulation of the lytic genes, including the immediate early transactivator Rta (383). However, in that study, the deletion did not significantly perturb latent gene expression or lytic replication, but was associated with an alteration in the virus's histone methylation and acetylation patterns and a reduction in the host's DNA methylation. Specifically, miR-K4–5p regulates DNA methylation via suppressing the host cell's retinoblastoma-like protein 2 (Rbl2), which is a negative regulator of DNMT3a/b. In contrast, Lei et al. (347) show that the same deletion did indeed enhance lytic replication, which was attributed to the deletion of miR-K1. Knockdown of miR-K1 is sufficient for inducing IkB$\alpha$ and, thus, inhibition of NF$\kappa$B (347). miR-K1 also targets the cell cycle inhibitor p21 (210), whereas miR-K5, miR-K9, and miR-K10 target bcl2-asociated factor 1 (BCLAF1) and reduce sensitivity to etoposide-induced apoptosis (718). Furthermore, KSHV miRNAs target musculoaponeurotic fibrosarcoma oncogene homolog (MAF) and induce dedifferentiation of endothelial cells (232). Interestingly, several herpes viruses v-miRNAs, including KSHV miR-K7, HCMV miR-UL112, and EBV miR-BART2–5p, target MHC class I polypeptide-related sequence B (MICB) at multiple sites as a means of evading natural killer cells (437). Collectively, these cellular targets may potentially contribute to the oncogenic capacity of KSHV.

One of the most intriguing KSHV miRNAs is miR-K11 (miR-K12–11), which is an ortholog of mammalian miR-155 (211, 564). Thus miR-K11 has the capacity to target the same cellular genes identified for miR-155 that are mentioned above and, accordingly, may play a role in the development of KSHV-induced B-cell lymphomas, which remains to be tested. Indeed, gene expression profiling revealed an overall overlapping pattern in cells expressing either miRNA (211, 564). In addition to miR-155 validated targets, miR-K11 was shown to target BTB and CNC homology 1, basic leucine zipper transcription factor 1 (BACH-1) (211, 564), c-Fos, XIAP associated factor 1 (BIRC4BP), angiotensin II receptor-associated protein (AGTRAP), SAM domain and HD domain 1 (SAMHD1), and riboflavin kinase (RFK) (211), while miR-K1, miR-K3–3p, miR-K6–3p, and miR-K11 target the tumor suppressor thrombospondin (525).

Similar to EBV, KSHV exploits the host cell's miRNAs to its advantage. Specifically, it induces upregulation of miR-146a via an NF$\kappa$B-dependent pathway, which then targets and downregulates chemokine (C-X-C motif) receptor 4 (CXCR4). This effect is predicted to induce premature release of KSHV-infected endothelial progenitor cells into the bloodstream, thus contributing to formation of Kaposi's sarcoma. Another miRNA that is induced by herpes viruses is miR-132 (326). It is upregulated after infection of lymphatic endothelial cells with KSHV and promotes viral replication via suppressing p300 and, in turn, interferon-stimulated genes.

### 3. Human immunodeficiency virus type 1

Similar to double-stranded DNA viruses, the single-stranded RNA human immunodeficiency virus type 1 (HIV-1) was predicted to harbor five pre-miRNA sequences (32). Indeed, direct cloning from HIV IIIB-infected MT-4 T cells and Northern blot verification confirmed the expression of a potential miRNA (miR-N367) from the *nef* gene (462). This gene is a conserved accessory gene that overlaps with the LTR and is expressed during viral replication. The data show that miR-N367 targets *nef* and reduces viremia, suggesting that it may play a role in reducing HIV-1 virulence. In addition, stem-loop transactivation responsive element (TAR) that is present at the 5′ end of all HIV-1 transcripts is processed by Dicer and produces two miRNAs, miR-TAR-5p and miR-TAR-3p (303, 465). These miRNAs are thought to inhibit viral transcription through recruitment of HDAC1 to the LTR promoter and, thus, plausibly promote latency. Further characterization of the function of these miRNAs and their targets in vivo is still needed.

The inhibitory effects of HIV-1 miRNAs on viral production reconciles well with the suppressive effect that both Dicer and Drosha have on viral replication (620). Bennasser et al. (30, 31) discovered that HIV-1's Tat protein interacts with Dicer and suppresses miRNA/siRNA processing. Furthermore, recent studies show that Vpr suppresses the expression of Dicer in macrophages (113). This antagonization of Dicer inhibits the processing of viral as well as cellular miRNA. In particular, the downregulation of cellular miR-17~92 was proven necessary for enhancing the virus's replication in Jurkat cells (620). One of the verified targets of miR-17–5p that explains its effect is the acetyltransferase p300/CBP-associated factor (PCAF) (620), which is required for Tat activity (297). Additionally, downregulation of miR-198 and upregulation of its target T cyclin is also

necessary for the virus's replication in monocytes (587). Intriguingly, several cellular miRNAs were also found to target a 1.2-kb region at the 3′ end of the viral genome and inhibit all viral gene expression and replication (249). These include miR-28, -125b, -150, -223, and -382, which are more abundant in resting versus activated CD4+ T cells. Notably, inhibiting these miRNAs in resting T cells from HIV-1-positive patients induced virus production. There is also evidence that elevated levels of these miRNAs render monocytes more resistant to HIV-1 infection (654).

In contradiction to a generalized reduction in miRNAs processing, miR-29a was found to be highly upregulated in HIV-1-infected human T lymphocytes (444). In this case, miR-29a interacted with the HIV-1's 3′-UTR and enhanced its association with RISC and P bodies, resulting in disruption of the latter. This led to enhanced virus replication and infectivity. This apparent inconsistency with the reported inhibition of Dicer can be explained by posttranscriptional regulation of miR-29a (291, 426, 618). Thus cellular miRNAs regulate viral replication indirectly through suppression of critical cellular proteins such as PCAF and T cyclin or disruption of P bodies, or directly through suppression of all viral proteins including TAT and Rev. These results demonstrate that certain cellular miRNAs play an essential role in regulating HIV-1 latency and may potentially serve as candidates for replacement therapy.

## IV. MicroRNAs IN THE HEART

### A. miRNAs in Cardiogenesis

Direct cloning (328) and microarray expression profiling (102, 533, 600, 626) have identified a large array of miRNAs that are expressed in the heart. In particular, miR-1 and miR-133 are highly enriched in the heart and the skeletal muscle and, thus, their roles in cardiac development have been the focus of several studies. miR-1 is highly conserved among species and the most abundant miRNA in the adult heart, where it represents ~45% of miRNA in the adult mouse heart (328) and ~24% in the adult human heart (358). Its expression coincides with the onset of cardiac myocyte differentiation in the developing heart tube and somitic myotomes in chick embryos (131) and in the looping mouse heart at embryonic day (E) 8.5, where it is regulated by SRF (713). Similarly, in *Drosophila,* dmiR-1 is expressed in the presumptive mesoderm during embryonic stage 5, and later in all its muscle cell derivates including the dorsal vessel (the mammalian heart counterpart) (568). Its function in *Drosophila* was uncovered in a homozygous knockout model, which showed that dmiR-1 is dispensable during muscle development, but is required for normal muscle growth and survival as the larvae transition from stage 1 to stage 2 (568). In contrast, its function in the rodent heart is essential during development, as perturbation of its normal levels by overexpression of a transgene, using a beta myosin heavy chain ($\beta$MHC) promoter, resulted in developmental arrest at E13.5. The observed defects included thin-walled ventricles and heart failure that is associated with reduced myocyte proliferation. Since miR-1 targets Hand2, it is likely that the phenotype is a consequence of an untimely reduction in endogenous Hand2 by excess miR-1 (713). Hand2, which is a transcription factor that appears in the straight heart tube of a mouse at E7.75, is critical for cardiac development (577). Targeted deletion of Hand2 is embryonic lethal at E11 and is associated with an absence of the right-sided ventricle, aortic arch arteries, trabiculae, and dilated aortic sac. The imperfect alignment between the effects of the reduction of Hand2 by overexpressing miR-1 versus genomic ablation suggests an involvement of additional miR-1 targets that modify the phenotype.

miR-1 functions were further addressed by genomic ablation of one (miR-1–2) of its two genes (miR-1–1 and miR-1–2). While this had no impact in the heterozygous animals, it was lethal between E15.5 and early neonatal life in the homozygous knockout mice (712). Since only one of two genes was deleted in this model, mature miR-1 continues to be produced by the second gene. However, because its levels were undetermined, the extent of reduction in mature miR-1 that elicited the phenotype is unknown. This is especially hard to estimate since quantitative differences in the transcriptional and posttranscriptional regulation between alleles is also unknown. The lethality observed in the homozygous mice was associated with ventricular septal defects in ~50% of embryos that died soon after birth, and pericardial edema and cardiac dysfunction in utero. In contrast to the phenotype observed in the *Drosophila*, there were no obvious skeletal muscle defects. However, since the level of miR-1 in the skeletal muscle of the knockout mice was undetermined, the possibility remains that the second gene might have compensated for any reductions. Furthermore, ~15% of the mice that escaped early lethality succumbed to ventricular dilatation and dysfunction within 2–3 mo. The remaining survivals had no major physical or contractile cardiac abnormalities, but there was frequent wall thickening due to hyperplasia that was accompanied by prolongation of the action potential and occasional sudden death. This is reminiscent of a conductivity defect and could be partly explained by the upregulation of miR-1's target Irx5 (712). Irx5 is a negative transcriptional regulator of the potassium channel Kcnd2/Kv4.2 that is expressed in an endocardial-to-epicardial gradient, responsible for an inverse expression pattern of Kv4.2, which creates a repolarization gradient in the myocardium (120).

miR-133 is another muscle-enriched miRNA, albeit its expression level in the heart is less than that of miR-1. While deletion of one or two miR-1 genes had a lethal phenotype, deletion of one of two genes of miR-133 expressed in the heart had no effect on cardiac development. In particular,

targeted deletion of miR-133a-1 or miR-133a-2, which each resulted in ~50% reduction in mature cardiac miR-133, equivalent to the decrease associated with cardiac hypertrophy, exhibited no cardiac growth or functional abnormalities under normal or pressure overload conditions (377). On the other hand, complete ablation of miR-133 via double gene knockout resulted in increased proliferation and apoptosis of myocytes, ventricular septal defects, and prevalent embryonic lethality, whereas those that survived developed severe cardiac dilatation and failure, but no associated hypertrophy (377). In contrast, with only ~25% of residual miR-133 that is expressed from the *miR133b* gene, skeletal muscle had no overt abnormalities. The lack of an effect in the skeletal muscle implies that either mature miR-133 is more than fourfold higher than saturating levels relative to its targets or that the effects of its reduction are not manifest under normal conditions. The latter explanation is more consistent with the function of miRNAs in general.

Interestingly, the phenotype of a βMHC-driven, cardiac-specific, miR-133 transgenic mouse was mostly similar to that of the miR-1 transgenic model described above. Specifically, the mice died in utero with reduced myocyte proliferation, thinning of the cardiac walls, and heart failure at E13.5 (377). miR-133 validated targets in this case include cyclin D2, which partly explains its effect on myocyte proliferation. In addition, serum response factor is also a validated target that was increased in the double-knockout hearts and might have contributed to the increase in smooth muscle actin observed in this model. Previous work has shown that overexpression of SRF in the heart leads to delayed-onset cardiac hypertrophy and reexpression of the fetal gene program at 24 wk of age that progresses to failure (708). In contrast, cardiac ablation of SRF results in developmental defects and lethality, associated with increased apoptotic cells (451, 474). Although the miR-133 knockout model exhibited an increase in SRF, it was not accompanied by myocyte hypertrophy, and in contradiction to SRF ablation, exhibited an increase in apoptotic cell death (377). Thus more miR-133 targets must be involved in the underlying pathology of the knockout phenotype.

Friend of GATA 2 (Fog-2) is also an important regulator of cardiac development whose deficiency results in embryonic lethality associated with ventricular and atrial septal defects and hypoplasia (589, 605). Recently, it was found that miR-130a directly targets and inhibits Fog-2. Unlike miR-1 and miR-133, Fog-2 is more widely expressed, with higher levels in the heart and lung than observed in kidney, liver, or brain (298). Consistent with the function of its target, forced expression of miR-130a in the heart resulted in a phenotype that is reminiscent of that induced by ablation of Fog-2 (298). Thus cardiac development is regulated by at least three miRNAs, miR-1, miR-133, and miR-130a, through an array of targets whose scope has not been fully discovered yet.

## B. miRNAs in Cardiac Hypertrophy

Early evidence that miRNAs were involved in cardiac pathology was attributed to the fact that they are differentially regulated during cardiac hypertrophy and failure in both rodent and human heart (102, 262, 407, 438, 533, 585, 600, 611, 626) **(FIGURE 3)**. The underlying pathogenesis in cardiac hypertrophy involves reprogramming of the gene expression profile to greatly resemble that of the fetal/neonatal heart (269, 280, 399). In agreement with a role of miRNAs in this switch, its expression pattern changes in accordance (499). One of the earliest changes observed after applying pressure overload on a mouse heart was the downregulation of miR-1, which preceded any other miRNA changes, the increase in cardiac mass, or contractile dysfunction (533). This suggested that miR-1 might be a cause rather than an effect of the underlying pathogenesis. Consistently, miR-1 is downregulated in genetic models of cardiac hypertrophy including the calcineurin (261) and AKT (76) transgenic hearts. In humans, miR-1 is decreased in the hypertrophied left ventricle of acromegalic patients (159) and in those with aortic stenosis (262), in which the ejection fraction is preserved (50–60%). In support of a general role in building muscle mass, miR-1 is also downregulated during hypertrophy of skeletal muscle (412). The significance of this reduction during myocyte hypertrophy was uncovered after normalizing its levels with exogenous miR-1, which completely suppressed growth-stimulated protein synthesis (533). On the other hand, knockdown of miR-1 in vivo in an adult mouse proved that it was also sufficient for induction of cardiac hypertrophy (76). Some of its predicted targets that have been validated include RasGAP, Cdk9, Rheb (only by Western blots) (533), insulin-like growth factor I (IGF-I) (159), calmodulin, and Mef2a (261), all of which play essential roles during cardiac hypertrophy.

In the failing human heart, the measurements of miR-1 levels were inconsistent, and accordingly, its function and targets in that context are less explained. While Matkovich et al. (407) and Thum et al. (611) report upregulation of miR-1, Ikeda et al. (262), Sucharov et al. (585), and Naga Prasad et al. (438) report a reduction in miR-1, in ischemic and nonischemic dilated cardiomyopathies accompanied by a significant reduction in percent ejection fraction. Overall, we may conclude that miR-1 functions in regulating the expression of genes that are essential for the increase in cell size and mass that characterize hypertrophy. However, as hypertrophy transitions into dilated cardiomyopathy and failure, miR-1 levels return to normal or above normal levels. This increase is also seen during ischemic heart disease, which will be discussed in the following section.

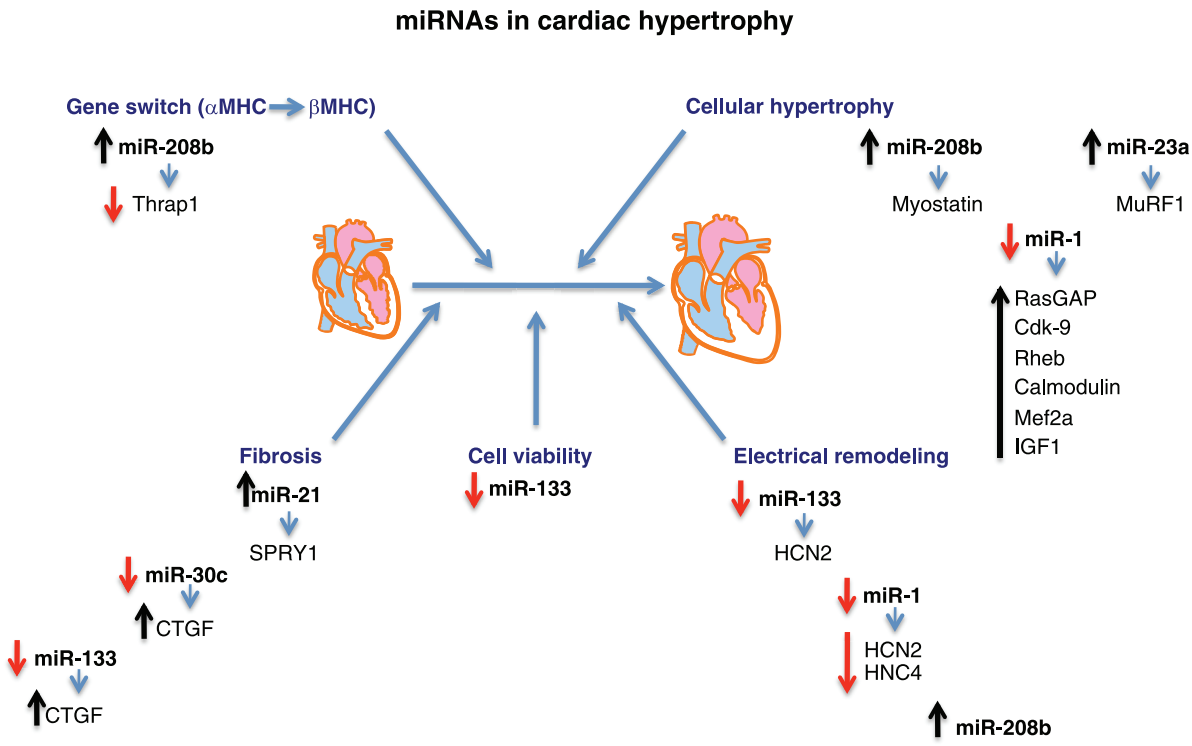


**FIGURE 3** A diagram showing miRNAs and their targets in cardiac hypertrophy. The diagram displays the different miRNAs and their targets that are involved in gene switching, cellular hypertrophy, fibrosis, and electrical remodeling during cardiac hypertrophy. Upregulation or downregulation of a specific miRNA is represented by an upward (black) or a downward (red) arrow, respectively. The changes in the expression levels of target genes inversely correlate with that of the targeting miRNA and are accordingly listed in red (downregulated) or black (upregulated) dialog boxes under the targeting miRNAs. All listed targets have been validated. The listed targets include the following: thyroid hormone receptor-associated protein 1 (Thrap1), myostatin, muscle-specific RING finger protein 1 (MuRF1), Ras GTPase-activating protein (RasGAP), cyclin-dependent kinase 9 (Cdk9), Ras homolog enriched in brain (Rheb), calmodulin, myocyte enhancer factor 2A (Mef2a), insulin-like growth factor 1 (IGF1), sprouty 2/4 (SPRY2/4), connective tissue growth factor (CTGF), hyperpolarization-activated, cyclic nucleotide-gated $K^+$ 2/4 (HCN2/4), and the indirect targets $\alpha$ and $\beta$ myosin heavy chain ($\alpha$- and $\beta$MHC).

In addition to miR-1, the other muscle-enriched miRNAs, miR-133, is also downregulated during cardiac hypertrophy (76, 102, 626). Knockdown of miR-133 via antisense targeting was sufficient for inducing cardiac hypertrophy and reinduction of the fetal gene program in the adult mouse heart (76). In contrast, though, targeted deletion of miR-133a-1 or miR-133a-2, which each resulted in ~50% reduction in cardiac miR-133, equivalent to the decrease associated with cardiac hypertrophy, exhibited no cardiac growth or functional abnormalities under normal or pressure overload conditions (377). The discrepancy between the outcomes could be explained by the temporal difference of miR-133 knockdown in the heart that is elicited by these two distinct approaches. A more definitive answer was derived by using a transgenic mouse model in which the overexpressed miR-133 compensated for its downregulation during hypertrophy (408). Although cardiac weight was not normalized as a result of this intervention, other aspects of hypertrophy that included apoptosis, fibrosis, and the downregulation of $I_{Kr}$, were restored to baseline levels, while the decline in $I_{to,f}$ and prolongation of Q-T interval, were partly recovered. However, miR-133 targets that mediate its effect under these conditions remain to be identified. Thus, while knocking down a gene in a normal cell background is an invaluable tool for understanding its function, restoring its level during a pathological condition is requisite for examining its function in that specific context. However, one has to also consider that overexpression of the transgene prior to inducing the pathology may have impacted the phenotype. These results are a clear indication that the outcome of manipulating the levels of endogenous miRNAs is highly dependent on the cell's transcriptome/proteome makeup.

While miR-1 and miR-133 are enriched in both skeletal and cardiac muscle, miR-208 is cardiac restricted (37). It has two isoforms, miR-208a and -208b, that have identical seed sequences, which diverge in only three nucleotides over the length of their mature sequence, and are, thus, predicted to have common targets (217, 218). Interestingly, these two genes are contained within the introns of *Myh6* ($\alpha$MHC) and *Myh7* ($\beta$MHC) genes, respectively. In the mouse heart, $\beta$MHC is expressed at high levels during early cardiogenesis and declines after birth, while $\alpha$MHC displays an inverse

pattern. During pressure-induced cardiac hypertrophy, an isoform switch results in recapitulation of the neonatal phenotype (517). Since the miR-208 a/b genes are coexpressed with their host genes (625), it is predicted that isoform switch will be paralleled by an equivalent switch in miR-208a/208b that is likely to sustain the level of total mature miR-208 and, thus, its targets under these conditions. However, while miR-208a level does not decrease within 3 wk of cardiac hypertrophy, miR-208b levels increased in parallel with βMHC (69), thus resulting in a net increase in miR-208. It was suggested that the lack of change in miR-208a levels is due to its relatively long half-life. In addition to miR-208, miR-499 is also contained within a myosin gene, *Myh7b,* whose transcription is constitutively high in the heart and slow-twitch skeletal muscle.

miRNAs that are enriched in skeletal and/or cardiac muscles have been appropriately dubbed myomiRs (411, 642). Their functions have been investigated by targeted genomic ablation in mice. Homozygous miR-208a mice (miR-208a$^{-/-}$) are developmentally normal with the expected normal levels of α- and βMHC at birth, while the adult mice failed to upregulate βMHC in response to thyroid hormone inhibition (627). Likewise, pressure overload on the heart did not induce the expected increase in βMHC. These effects are thought to be mediated by thyroid hormone receptor-associated protein 1 (THRAP1), which is a validated target of miR-208 that is upregulated in the knockout mouse. THRAP1/MED13/TRAP240 is involved in the transcriptional regulation of a wide array of genes, including those regulated via nuclear receptors (308, 527, 699). In agreement, a transgenic mouse model overexpressing miR-208a in the heart reduced THRAP1 and induced an increase in βMHC, suggesting that endogenous miR-208a is below saturating levels (69, 627). In addition to the lack of responsiveness to thyroid hormone inhibition in the miR-208a$^{-/-}$, there was absence of hypertrophic growth and fibrosis following an increase in work load, in addition to reduced levels of miR-499 (627). Interestingly, overexpression of miR-499 in the miR-208a$^{-/-}$ model restored the normal response to thyroid hormone inhibition, suggesting that it is a downstream effector of miR-208 and is sufficient for mediating its effect. Paradoxically, though, ablation of miR-499 did not recapitulate the miR-208a$^{-/-}$ phenotype (625).

Like miR-208a$^{-/-}$, miR-208b$^{-/-}$ mice were also developmentally normal. In line with the expression pattern of their host genes, miR-208a is expected to be the major source of mature miR-208 in the adult mouse heart, while miR-208b is the major source in the developing heart. Accordingly, ablation of the latter provides a more definitive conclusion regarding the lack of a role for miR-208 in cardiac development. Moreover, the heart of a double miR-499/miR-208b knockout was also normal, which contrasted with the lack of βMHC and type I myofibers in the soleus muscle. Sox6, Sp3, and Purb were identified as likely candidates in mediating the suppression of βMHC expression in the absence of miR-208a or miR-499. Thus, while miR-208 is not necessary for the expression of βMHC during cardiac development, it is required for its upregulation during cardiac hypertrophy and hypothyroidism **(FIGURE 3)**.

In contrast to the lack of a phenotype in the miR-208a/b knockout models, overexpression of miR-208a in mice induced cardiac hypertrophy that was only manifest after 4 mo of age (69). This phenotype, though, was not associated with a decrease in αMHC, an increase of ANF, or a change in any of the hypertrophy-associated miRNAs. In contrast, myostatin, which is a negative regulator of hypertrophy (430), is a validated target of miR-208a that was suppressed in the transgenic heart and, thus, might explain the hypertrophic growth (69). These mice also exhibited defects in conduction in the form of first- or second-degree atrioventricular bundle block, compared with the miR-208a$^{-/-}$ mice, which displayed atrial fibrillation. As a possible mechanism, connexin40 (Cx40) and its transcriptional regulator Hop were dramatically reduced in the miR-208a$^{-/-}$ mice, although they were unaffected in the transgenic model. Thus modulation of these proteins could potentially explain the conduction defects in the former but not the latter model. Meanwhile, GATA4, a positive regulator of Cx40 (371) and a proven target of miR-208a, is upregulated in miR-208a$^{-/-}$ mice; however, this does not reconcile with the observed downregulation of Cx40.

While the focus in this section was on the roles of miR-1, miR-133, and miR-208 in cardiac hypertrophy, the differential display studies have revealed an abundance of other miRNAs that are also dysregulated that are apt to play a role in the underlying pathogenesis **(FIGURE 3)**. However, not many have received as much attention as did the myomirs. miR-21 is one of the most highly and consistently upregulated miRNA during cardiac hypertrophy (102, 533, 600, 626), whose role in hypertrophy has been elusive, as a transgenic mouse overexpressing the gene in the heart was completely normal and its response to pressure overload hypertrophy was comparable to wild-type mice (612). While miR-21 is upregulated in the whole heart during pathological hypertrophy, it appears that its increase in the myofibroblasts is more significant than it is in the myocytes (612). Through targeting and suppressing sprouty1, it enhances erk1/2 phosphorylation and myofibroblast survival (612). This effect indirectly contributes to the increase in fibrosis seen during cardiac hypertrophy. Accordingly, antisense targeting of miR-21 reduced fibrosis and hypertrophy. In addition, miR-21 also targets sprouty2 via which it increases connexin43-positive junctions between myocytes, facilitating the exchange of small molecules between cells (534). Since pathological hypertrophy is associated with a rise in myocyte apoptosis, it is plausible that the increase in miR-21 is also protective of cardiac myocytes under this condition. Indeed, miR-21 displays an antiapoptotic func-

tion in cardiac myocytes during myocardial ischemia, as will be described in the next section.

miR-23a, which is clustered with miR-27a and miR-24, is also upregulated during pressure overload (102, 533, 600, 626) and isoproterenol-induced hypertrophy (370). It is directly regulated by NFATc3 and is both necessary and sufficient for mediating the hypertrophic effect of the calcineurin-NFATc3 pathway. It exerts its effects via targeting and suppressing the negative regulator muscle ring finger 1 (MuRF1). In accordance, knockdown of miR-23a in the mouse heart using antisense antagomirs results in an increase in MuRF1 and near-complete inhibition of isoproterenol-induced cardiac hypertrophy, including changes in molecular markers and cardiac function. This reconciles with the phenotype observed of the MuRF1 knockout mice, which develop more pronounced pressure-induced hypertrophy (664).

## C. miRNAs in Ischemic Heart Disease

Myocardial ischemia is a result of insufficient blood supply that leads to apoptotic, autophagic, and necrotic cell death, and subsequent fibrosis of the heart that impairs contractility, and is the leading cause of mortality in developed countries. miRNAs levels are rapidly perturbed upon exposure of cells or organs to hypoxia or ischemia **(FIGURE 4)**. In contrast to cardiac hypertrophy, miR-1 and miR-133 are upregulated during exposure of myocytes to oxidative stress, where miR-1 was found to target heat shock proteins (HSP) HSP60 and HSP70, and miR-133 targets caspase-9 (679). Accordingly, miR-1 exerts proapoptotic effects, while miR-133 is antiapoptotic. On the other hand, miR-320 is downregulated during ischemia/reperfusion in the heart and is responsible for upregulation of HSP20 (507). Further reduction of endogenous miR-320 with an antisense construct successfully reduces infarct size after ischemia reperfusion. Meanwhile, miR-21, which is ubiquitously expressed, has emerged as a critical regulator of cell apoptosis in both myocytes and nonmyocytes in the myocardium. It is acutely reduced in the heart during ischemia, specifically within the ischemic region, where replenishing it reduces infarct size (147, 532). In contrast, it is upregulated in the periinfarct zone where the myocytes are undergoing hypertrophy. This rescue was seen in a cardiac-specific transgenic model of miR-21 (532), as well as via local viral delivery

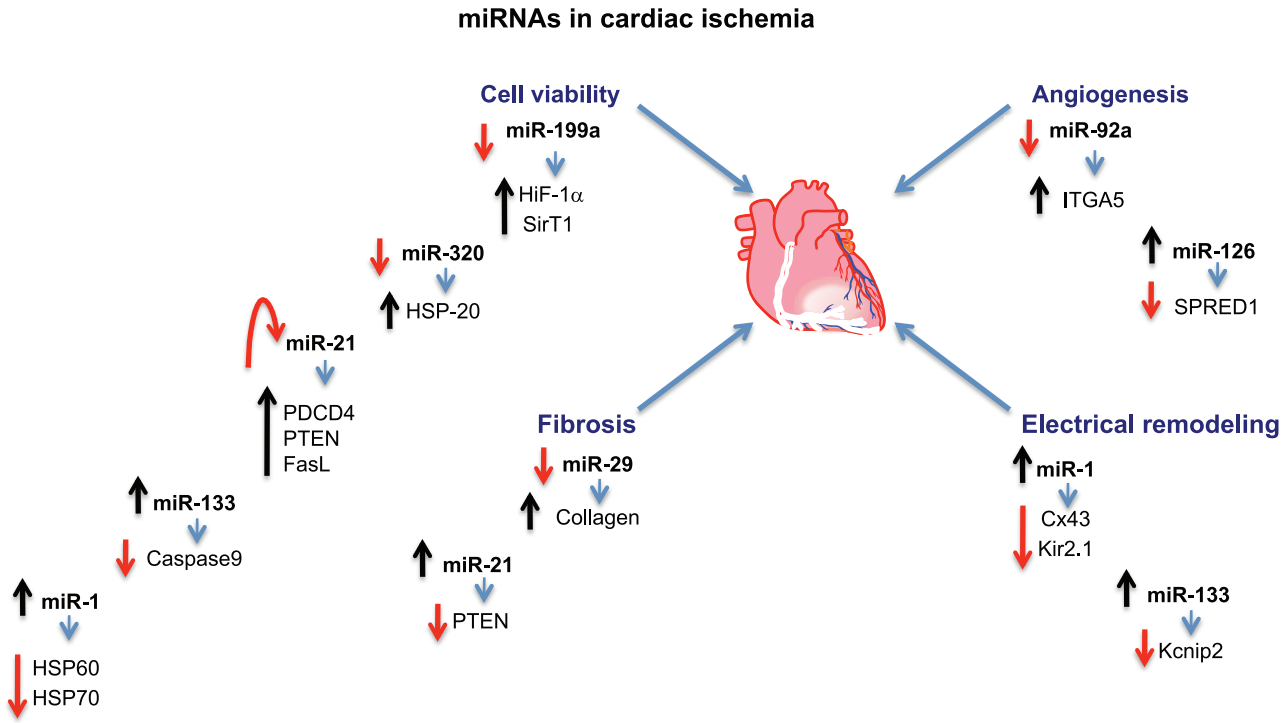


**FIGURE 4** A diagram showing miRNAs and their targets in cardiac ischemia. The diagram displays the different miRNAs and their targets that are involved in cell viability, angiogenesis, fibrosis, and electrical remodeling during cardiac ischemia. Upregulation or downregulation of a specific miRNA is represented by an upward (black) or a downward (red) arrow, respectively. The changes in the expression levels of target genes inversely correlate with that of the targeting miRNA and are accordingly listed in red (downregulated) or black (upregulated) dialog boxes under the targeting miRNAs. The listed targets include hypoxia-inducible factor 1 $\alpha$ (Hif-1$\alpha$); sirtuin 1 (Sirt1); heat shock proteins 20, 60, and 70 (HSP20, HSP60, HSP70); programmed cell death 4 (PDCD4); phosphatase and tensin homolog (PTEN); fas ligand (FasL); caspase 9; integrin $\alpha$ 5 (ITGA5); sprouty-related EVH1 domain containing 1 (SPRED1); connexin 43 (Cx43); inward rectifier potassium channel 2 (Kir2.1); and cardiac voltage-gated potassium channel modulatory subunit (Kcnip2).

of that miRNA (147). These results were further confirmed in isolated cardiac myocytes, in which exogenous miR-21 was shown to inhibit apoptosis by targeting Fas ligand (FasL) (532) or programmed cell death 4 (PDCD4) (104). Interestingly, miR-21 is a downstream effector of AKT that partly mediates its antiapoptotic effect via suppression of FasL (532).

miR-21 is also enriched in the myofibroblasts and infiltrating cells in the infarct zone relative to the surrounding myocytes. In these cells, it inhibits PTEN and induces upregulation of metalloprotease-2 (515), which promotes fibrosis. As mentioned above, miR-21 is also profibrotic during cardiac hypertrophy, via enhancing myofibroblast survival (612). However, the involvement of this mechanism in myocardial ischemia has not been explored yet. In addition to miR-21, downregulation of miR-29, which targets multiple collagen isoforms (628), may potentially contribute to the development of fibrosis during ischemia. In support, antisense knockdown of miR-29b in a mouse model confirmed that it is sufficient for inducing an increase in collagen in the heart. Thus precise replenishment of miR-29 in the ischemic heart may be a potential therapeutic advantage. On the other hand, since miR-21 has both antiapoptotic and profibrotic effects, its therapeutic targeting has to be carefully evaluated.

The response of a cell to hypoxia or ischemia is bimodal, in the form of an initial, adaptive, conditioning reaction that transitions into cell death upon persistence of the insult. Accordingly, brief exposures to hypoxia or ischemia elicit an antiapoptotic response that involves activation of the AKT pathway (237), which subsequently protects the cells against protracted periods of hypoxia or ischemia that may immediately follow. This is known as early hypoxia preconditioning (HPC), or ischemia preconditioning (IPC) (436). miRNAs are also involved in regulating this process, which requires translational but not transcriptional events (514), including upregulation of hypoxia-inducible factor 1$\alpha$ (Hif-1$\alpha$) (65, 154). Specifically, miR-199a-5p is acutely downregulated through a posttranscriptional mechanism, in cardiac myocytes and the heart, following HPC or IPC, respectively. In myocytes, this downregulation proved both necessary and sufficient for the rapid upregulation of its target Hif-1$\alpha$ (497). Sirt1 is also a direct and validated target of miR-199a-5p that complements it function by downregulating prolyl hydroxylase 2 and, thereby, stabilizing Hif-1$\alpha$ (497). These effects are mediated by AKT, which induces upregulation of Hif-1$\alpha$ and Sirt1 via of downregulation of miR-199a-5p (498). Thus miR-199a-5p is an example in which a miRNA functions as an immediate early switch that enhances the translation of a gene whose expression is required for an acute response. In contrast, AKT induces upregulation of miR-21 (532), which is also increased following ischemia preconditioning of the heart (105, 694). Through targeting major antiapoptotic genes such as PTEN, FasL, and PDCD4, it assumes a powerful antiapoptotic function during ischemic conditions. Thus the AKT pathway utilizes miRNAs to both increase prosurvival proteins and suppress proapoptotic molecules during early ischemia preconditioning.

In addition to miRNAs-regulated apoptotic and survival pathways involved in myocardial ischemia, miRNAs also regulate angiogenesis and, thus, influence the rate of tissue healing after ischemia. Please refer to the section on angiogenesis for a description on the role of miRNAs in this vital process.

### D. miRNAs in Electrical Remodeling of the Heart

Arrhythmias are characteristic of coronary/ischemic heart disease, where they are elicited by abnormalities in electrical conduction and/or repolarization. As miRNAs regulate a cell's response to ischemia, they also regulate the expression of molecules involved in mounting an action potential, as well as the cell's conductivity. miR-1 is increased in the human hearts that have suffered coronary artery disease (CAD) and in rat ischemic hearts, which are associated with an increase in arrhythmogenesis (686). Increasing or decreasing miR-1 in the ischemic rat heart results in an equivalent effect on the frequency of arrhythmias. miR-1 targets that could explain its effects include the gap junction protein Cx43 and Kir2.1, which is a subunit of the $K^+$ channel that mediates $I_{K1}$. Inhibition of arrhythmias by knockdown of endogenous miR-1 is reversed by concomitant knockdown of both these molecules. In support, ablation or missense mutation of Cx43 (349) or $I_{K1}$ (489), respectively, are associated with increased incidence of arrhythmias. In contrast to their regulation in ischemic conditions, miR-1 and miR-133 are downregulated during cardiac hypertrophy, where miR-1 targets HCN2 and HCN4, and miR-133 targets HCN2 (395). These proteins are components of the pacemaker channel responsible for the hyperpolarization-activated current ($I_f$). They are also upregulated during cardiac hypertrophy and failure and have been implicated in arrhythmogenesis (425). The downregulation of miR-133 during cardiac hypertrophy is also associated with a decrease in the $I_{to,f}$ accessory subunit *Kcnip2*, albeit via an indirect mechanism, and is, accordingly, responsible for prolongation of the QT interval (408). Thus miR-1 and miR-133 are major regulators of various disease aspects in both the hypertrophic and ischemic heart through a broad spectrum of targets (Figures 3 and 4).

## V. MicroRNAs IN SKELETAL MUSCLE

### A. miRNAs in Myogenesis

miRNAs are intricately involved in self-renewal and differentiation of myogenic stem cells (MSC) **(FIGURE 5)**. MSC

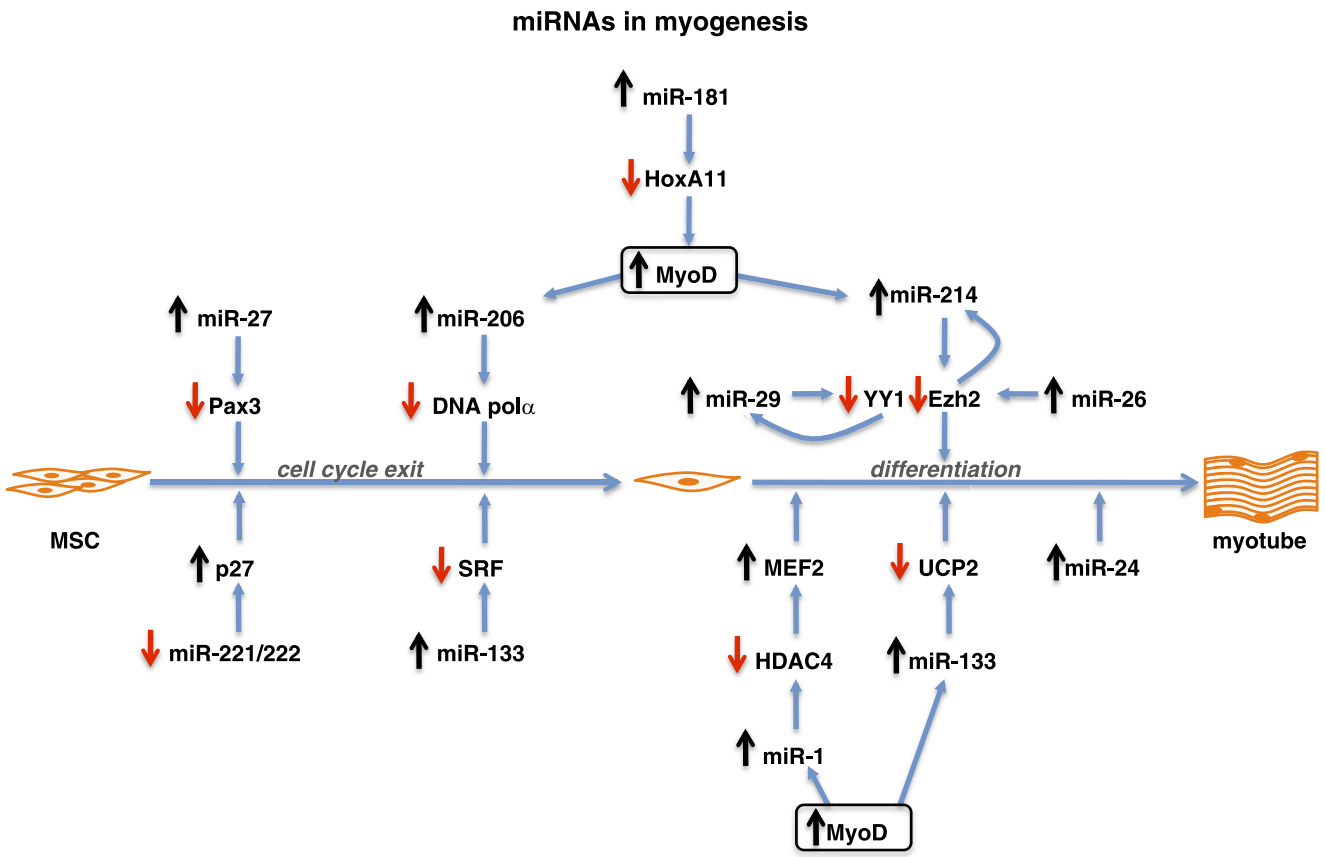


**FIGURE 5** A diagram showing miRNAs and their targets in myogenesis. The diagram displays the different miRNAs and their targets that are involved in cell cycle exit of myogenic stem cells (MSC) and their differentiation into myotubes. The muscle-specific transcription factor MyoD plays a central role through regulating the expression of the muscle-enriched miR-1, miR-133, and miR-206. Upregulation or downregulation of a specific miRNA is represented by an upward (black) or a downward (red) arrow, respectively. The changes in the expression levels of a target gene inversely correlate with that of the targeting miRNA and are similarly represented by an up or down arrow. Only the miRNA targets that have been specifically verified in skeletal muscle are listed. All listed targets have been validated. The listed targets include homeobox A11 (HoxA11), paired box gene 3 (Pax3), DNA polymerase alpha catalytic subunit (DNA pola), serum response factor (SRF), zeste homolog 2 (Ezh2), ying and yang 1 (YY1), histone deacetylase 4 (HDAC4), and uncoupling protein 2 (UCP2).

maintain their self-renewal capacity in part through the transcription factor Pax3 (13). Thus downregulation of Pax3 is necessary for exiting the cell cycle and myogenic differentiation. miR-27 was identified as a targeting miRNA of this molecule and is accordingly expressed in differentiating skeletal muscle in both the myotome and adult satellite cells (127). A transgenic mouse model confirmed that ectopic expression of miR-27 downregulates Pax3 and induces premature differentiation of progenitor cells. Conversely, knockdown of miR-27 delays differentiation, which is consistent with the effect of sustained expression of Pax3 (57). While Pax promotes proliferation, the polycomb group (PcG) suppresses differentiation by occupying the MyoD enhancer site and inhibiting MyoD-dependent transcription (343). The zeste homolog 2 (Ezh2) is a histone lysine methyltransferase subunit of this complex that mediates promoter silencing through H3K4 and H3K27 methylation. In accordance, Ezh2 is present in myotomes and is downregulated upon differentiation, allowing MyoD to bind to and activate muscle genes (77). Interestingly, PcG was found to occupy a MyoD enhancer site upstream of miR-214 in undifferentiated muscle cells (285). Upon differentiation, PcG is released and MyoD induces upregulation of miR-214, which creates a negative feedback loop by targeting and inhibiting Ezh2 (285). Accordingly, overexpression of miR-214 accelerates C2C12 differentiation. Similar results were observed with miR-26a, which also targets Ezh2, although in this case it is not known whether MyoD regulates its expression (666). Notably, Ezh2 requires the transcriptional regulator YY1 for binding to, and blocking, MyoD enhancers (343). miR-29 targets and suppresses YY1, which in turn inhibits miR-29 in a negative feedback loop (666). Thus upregulation of miR-214, -26, and -29 is collaboratively involved in suppressing PcG expression and function in muscle stem cells and, thereby, promoting muscle-specific gene expression and differentiation. In addition, upregulation of miR-181 promotes differentiation by suppressing a MyoD negative regulator, HoxA11 (439), while downregulation of miR-221/222 derepresses $p27^{kip1}$, inhibiting proliferation (75).

Upon initiation of differentiation, MSC become enriched in miR-206, miR-1, and miR-133, plausibly via myoD-dependent transcriptional regulation (501). While both miR-1 and miR-133 simultaneously increase during differentiation of C2C12, they were reported to have antagonistic effects (91). miR-1's expression is necessary and sufficient for inducing differentiation (91, 441), whereas miR-133 expression suspends differentiation via sustaining proliferation (91). Their relevant targets in this context are HDAC4 and SRF, respectively, where HDAC inhibits the transcriptional activity of the muscle specific Mef2c, and SRF inhibits proliferation of myoblasts. The relevance of having these two opposing but overlapping functions during muscle differentiation is not readily explainable, since the accelerated differentiation induced by overexpression of miR-1 has not been examined in the absence of miR-133, or vice versa. In contrast, though, another study showed that upregulation of miR-133 is essential for downregulation of uncoupling protein 2 (UCP2), which is an inhibitor of muscle differentiation (96). UCP1 is a mitochondrial transporter that uncouples ATP synthesis. Although UCP2 is highly homologous to UCP1, its primary function remains unknown. The UCP2 is an example of a gene whose mRNA but not its translational product is found in the heart, which is consistent with the fact that it is a target of the muscle-enriched miR-133. Its inhibition, but not that of UCP3, proved necessary for C2C12 differentiation.

In contrast to the effect of miR-1 and miR-133 in cultured cells, genomic knockout of both miR-133a-1 and miR-133a-2 (377) or miR-1–2 (712) had no apparent impact on skeletal muscle development in the surviving mice. In contrast, disruption of miR-133 or miR-1 in zebrafish resulted in reduced fiber size, while knockdown of both disrupted sarcomeric actin organization (428). The phenotype of the miR-1–2 knockout mouse model could be explained by compensatory upregulation of the second allele of miR-1 (miR-1–1) or miR-206, which have the same seed sequence. On the other hand, genomic ablation of miR-133a resulted in more than 70% reduction of endogenous mature miR-133, with no apparent compensation from miR-133b, suggesting that miR-133 had no role in muscle differentiation or growth in vivo. Thus the role of miR-1 and miR-133 in muscle development in vivo needs a more detailed examination. On the other hand, there is evidence that downregulation of miR-1/206 is associated with muscle hypertrophy, as the levels of miR-1 and miR-133 are ~50% lower in hypertrophied skeletal muscle in mice (412), similar to what is observed in cardiac hypertrophy (76, 533).

While miR-1 and miR-133 are also expressed in the heart, miR-206 is a skeletal muscle-restricted miRNA, which, in consensus, is induced by MyoD (513). Its mature sequence differs in only three nucleotides from that of miR-1, which are outside the seed sequence. Thus the predicted targets of miR-1 and miR-206 are the same. Accordingly, it is not surprising to find that like miR-1, overexpression of miR-206 enhances differentiation of C2C12 cells (299). Moreover, like miR-1 and miR-133, ablation of miR-206 in a mouse model had no effect on normal muscle development (663). Additional direct targets that were validated for miR-206 include the p180 subunit of the DNA polymerase α-subunit (299), whose downregulation coincides with cell cycle exit and differentiation of various tissues (252). Although Cx43 is a predicted and validated target that is suppressed by miR-1/206 during C2C12 differentiation, this finding does not agree with many studies that show that Cx43 is also critical for myoblast differentiation (14, 15, 494). It could be envisioned, though, that the ratio of miR-206 to Cx43 may fine-tune the expression of Cx43 during different stages of muscle development; thus more detailed dose- and time-dependent experiments are required for clarifying the role of miR-206 in regulating Cx43. miR-206 also directly targets and suppresses the expression of utrophin and follistatin (513), which are required in formation of the postsynaptic fold (138) and stimulation of angiogenesis (464), respectively, but have no known direct roles in myogenesis yet.

Thus, at least in cultured skeletal myotubes, miR-1, -133, and -206 are positively involved in myogenic differentiation through the regulation of various aspects of this process. Ishikawa et al. (442) exploited this functionality by locally applying a mixture of these miRNAs to an injured rat tibialis anterior muscle, where they observed enhanced muscle regeneration and less fibrosis. Indeed, this could provide future therapeutic utility during muscle injury.

## B. miRNAs in Muscle Pathologies

### 1. Muscular dystrophies

Expression profiling of miRNAs revealed 185 differentially expressed miRNA in 10 major muscular disorders in humans including Duchenne muscular dystrophy (DMD), Becker muscular dystrophy (BMD), facioscapulohumeral muscular dystrophy (FHMD), limb-girdle muscular dystrophies types 2A and 2B (LGMD2A and LGMD2B), Miyoshi myopathy (MM), nemaline myopathy (NM), polymyositis (PM), dermatomyositis (DM), and inclusion body myositis (IBM). Interestingly, five miRNA only (miR-146b, miR-221, miR-222, miR-155, and miR-214) were consistently upregulated in all forms of MD except BMD, which did not include any of the muscle-enriched myomiRs (miR-206, miR-1, miR-133). Another interesting profiling was one that compared DMD samples or dystrophin-deficient mice (mdx) with acute ischemic muscle degeneration and regeneration phases (214). The miRNAs were accordingly classified into three categories that included the following: regeneration miRNA (miR-31, -34c, -206, -335, and 449) that were upregulated in DMD/mdx and postischemic regeneration, degeneration miRNA (miR-1, -29c, and -135a)

that were downregulated in DMD/mdx and postischemic degenerative phase, and inflammatory miRNA (miR-222 and -223) that were increased in the damaged, infiltrated, regions. However, caution has to be exercised in interpreting these data, as McCarthy et al. (413) have previously shown that miR-206 is not uniformly upregulated in the different skeletal muscles of mdx mice. Also, the functional relevance of these changes during muscle regeneration awaits investigation.

#### 2. Rhabdomyosarcoma

This form of sarcoma originates from a muscle cell lineage. One of its characteristic is the increased expression of c-Met, a tyrosine kinase receptor activated by hepatocyte growth factor (178, 505). miR-1/miR-206 are reduced to undetectable levels in rhabdomyosarcoma (RMS) and have been shown to directly target c-Met, suggesting that they contribute to the upregulation of this protein (601, 684). Indeed, overexpression of either miRNA in RMS cells resulted in reduced proliferation, migration, and increased apoptosis. In addition, it induced a shift in the gene expression profile of RMS cells towards that of a differentiated muscle (601). More conclusively, ectopic expression of either miRNA in implanted tumor cell inhibited tumor growth (601, 684). Similarly, YY1, a cancer-associated transcription factor (79), is also increased in RMS and is involved with miR-29 in a double negative regulatory loop (647). In accordance, overexpression of miR-29 in implanted RMS cells resulted in retardation of tumor growth.

#### 3. Neurodegenerative diseases

Denervation induces differential dysregulation of muscle miRNA in the form of downregulation of miR-1 and miR-133 within 1 wk in rat soleus muscle, which returns to normal levels during the reinnervation phase (272). On the other hand, miR-206 is initially unchanged but increases during reinnervation. Although genomic ablation of miR-206 had no impact on normal muscle or neuromuscular junction development, it did provide evidence that miR-206 is involved in accelerating the reinnervation process (663). This was further substantiated by the fact that the absence of miR-206 accelerated muscle atrophy in an amyotrophic lateral sclerosis (ALS) mouse model (expressing an activated mutant of supreoxide dismutase). miR-206 targets and inhibits the expression of HDAC4, which is accordingly increased in miR-206 knockout (KO) relative to denervated wild type muscle. Thus, as predicted, muscle-specific knockout of HDAC4 enhanced muscle reinnervation. It was suggested that these effects might be mediated by fibroblast growth factor binding protein 1, which is downregulated in the miR-206 KO and upregulated in the HDAC4 KO mice, although its role in this process remains to be elucidated.

## VI. miRNAs IN THE BRAIN

### A. miRNAs in Neurogenesis

Knockout of Dicer provided unequivocal evidence that miRNAs play a role in brain development. Using an *Emx1* gene promoter that is specifically expressed in neuroepithelial cells in the dorsal telencephalon starting at embryonic day 9.5, or a *CAMKII* promoter that is expressed in excitatory neurons of the cortex, hippocampus, and caudate-putamen starting at E15.5, Cre-lox-dependent ablation of dicer in both models resulted in a smaller cortex and premature death around P21 (134, 137). This could be partly explained by an increase in programmed cell death that was observed in the cortex. In addition, the former model was associated with a 30% reduction in the neuronal layer and number of neurons at E13.5 (137), while the latter model was associated with a reduction in dendritic branching, an increase in dendritic spine length, and ataxia (134). Intriguingly, though, the thickness of the progenitor layers, progenitor cell proliferation, development, and differentiation were normal during early neurogenesis (137). The subsequent reduction in mitotic cells in the ventricular zone (VZ) and subventricular zone (SVZ) at E14.5 was attributed to an increase in cell death. Apoptosis is also a feature of postnatal knockout of dicer in differentiated Purkinje cells, using Pcp2 promoter-Cre recombinase, which is associated with cerebellar degeneration and ataxia (535), plausibly due to downregulation of ataxin1-targeting miR-19, miR-101, and miR-130 (345). The results suggest that while miRNAs are not required for cell differentiation, they are involved in cell homeostasis and survival.

miR-124 is a brain-enriched miRNA (328), where it starts increasing at E13 and remains elevated throughout adulthood (317, 567). In particular, it is localized to differentiated neurons (567) and is suppressed in non-neuronal cells by the RE1-silencing transcription factor (REST) (114). In agreement with the phenotype of Dicer ablation, ectopic expression or knockdown of miR-124 in the chick neural cord, including the neuronal progenitor cells, had no impact on neuronal differentiation or neurogenesis (74). However, its overexpression was associated with some cell death and disruption of the basal lamina that encloses the neural tube, via suppression of laminin $\gamma$1 and integrin $\beta$1. The latter is consistent with the fact that in some cases miRNAs and their predicted targets have evolved to avoid coexistence (170). Thus ectopic expression of miR-124 inhibits expression of its targets laminin $\gamma$1 and integrin $\beta$1, which are highly expressed in the ventricular zone progenitor cells but minimally in the high miR-124-expressing mantle zone. Controversially, another study showed that overexpression of miR-124 enhanced neurogenesis in the same model via suppression of synaptonemal complex protein 1 (SCP1), an anti-neural factor that is recruited by REST (637). The difference between studies could be explained by the fact that

miR-124 in the subventricular zone stem cells niche regulates the timing of differentiation of transit amplifying cells into neuroblasts and neuroblasts into neurons, where it is upregulated at each of these steps (101). Furthermore, knockdown of miR-124 delayed, but did not inhibit, differentiation of progenitor cells during neuronal regeneration.

While miR-124 had no independent effect on stem cell differentiation, it did collaborate with miR-9 to preferentially inhibit the differentiation of progenitors cells into glial cells, while increasing the ratio of neuron to glial cells (318). miR-124 was found to target and inhibit distal-less homeobox 2 (Dlx2) and Jagged 1 (Jag1), which play roles in interneuron formation and self-renewal of SVZ, respectively (101). Another highly relevant target is Sox9, which is known to be involved in promoting glial cell specification (581). In agreement, overexpression of miR-124-resistant Sox9 in SVZ cells delayed neurogenesis as it maintained the astrocyte population. miR-124 also exerts its function by inducing an alternative splicing pattern of genes that is characteristic of neurons (401). It exerts this effect through targeting and inhibiting the splicing suppressor polypyrimidine tract binding protein 1 (PTBP1), which results in alternative splicing of exon 10 of PTBP2 and an increase in its protein. In addition to its role in neurogenesis, miR-124 may be required for neurite outgrowth through suppression of Cdc42 and relocalization of Rac1, as seen during the differentiation of P19 cell (697).

miR-9 is also enriched in the brain (328), as it gradually increases during the development of the mouse brain, but unlike miR-124, it declines after birth (317, 567) and exists in both neurons and astrocytes (567). Except for subtle abnormalities in the sensory organs in *Drosophila,* it is dispensable for this species' normal development and survival (367). In particular, its ablation resulted in extra ddaE and ddaF neurons in the third instar larvae and extra sensory bristles on the anterior wing margins of adult flies, suggesting that it is required for controlling the precise number of sensory organs generated. This is consistent with a role of miRNAs in fine tuning cellular processes versus essentiality. On the other hand, in zebrafish, miR-9 is necessary for establishing the midbrain-hindbrain boundary (351). In the developing mouse brain, at E10.5, miR-9's expression is dorsomedially graded in the cortex, and at E12.5 it becomes enriched in the most medial pallium (558). Intriguingly, Foxg1, which suppresses differentiation of neuroblasts into Cajal-Retzius cells (231), is absent in this region and has been validated as a miR-9 target. Cajal-Retzius cells are the earliest born neurons in the neocortex that arise from the cortical hem. In vivo gain- and loss-of-function of miR-9 in the developing mouse brain ascertained that it was critical for the differentiation of progenitor cells into Cajal-Retzius cells (558). Although overexpression of miR-9 in neuronal stem cells was not sufficient for inducing differentiation, it did accelerate the process in the presence of differentiating medium, leading to an increase in both glial and neuronal cells in vitro and in vivo through targeting tailess (TLX) (711), a nuclear receptor involved in adult neural stem cell self-renewal (329). miR-125 is also one of the miRNAs that is highly expressed in the brain and has a similar expression pattern as miR-9 during the development of the mouse brain (567). However, in contrast, it was reported to be both necessary and sufficient for inducing differentiation of a human neuroblastoma cell line as well as a human neuronal progenitor cell line (335).

miR-134 is another brain-restricted miRNA, increasing gradually in the hippocampus, where it plateaus at P13 after synaptic maturation and becomes localized to dendrites near synaptic sites (537). Overexpressing it in hippocampal cells decreases the volume of dentritic spines and vice versa, an effect that is mediated by Lim domain-containing kinase-1 (LIMK-1). In agreement, a knockout model of this protein exhibits a similar phenotype (418). The dendritic spines are known to increase in size during synaptic excitation and, thereby, establish sites of synaptic contact. It is proposed that, upon brain-derived neurotrophic factor (BDNF) stimulation, miR-134 is released from LIMK-1 3′-UTR, resulting in upregulation of the protein, which increases spine volume and, thus, establishment of synaptic contact (537). In addition, a differential display revealed other miRNAs that are enriched in synaptosomes versus forebrain, which include miR-9 and miR-138 (561). Similar to miR-134, miR-138 is a negative regulator of spine volume and synaptic transmission, in this case through targeting acyl-protein thioestrase 1, known to regulate synaptic strength (157). On the other hand, BDNF stimulation of cortical neurons induced upregulation of miR-132, but not miR-134 or miR-138, which was required for upregulation of the glutamate receptors through an indirect mechanism (293). One of its previously validated targets include methyl CpG-binding protein 2 (MeCP2), which is the protein mutated in Rett syndrome (304); however, its role in this context remains unknown.

## B. miRNAs in Neurodegenerative Diseases

### 1. Alzheimer's disease

Mutations in amyloid precursor protein (APP), presenilin 1, and presenilin 2 are associated with familial Alzheimer's disease (AD). APP is cleaved by the β-amyloid cleavage enzyme (BACE1), generating the C99 product, which is then cleaved by the γ-secretase complex (includes presenilins) into Aβ40 and Aβ42 (reviewed in Ref. 545). Accumulation of β-amyloid plaques is a hallmark of AD; thus there has been much focus on miRNAs that regulate the proteins involved in this process. AD has a distinct miRNA expression pattern, where miR-9, miR-125b, and miR-146a are upregulated in the temporal lobe neocortex of AD patients but not in those with Parkinson's, schizophrenia, or ALS

(549). In contrast, miR-29b is downregulated in sporadic AD patients and inversely correlates with BACE1 expression (241). It directly targets BACE1 and suppresses its expression and, thereby, proteolytic cleavage of APP. Similarly, miR-107 is downregulated during early stages of AD and also directly targets BACE1 (653). Although only confirmed in a mouse model of AD, downregulation of miR-298 and miR-328 also plays a role in regulating BACE1 through direct targeting (51). On the other hand, APP is a direct target of miR-106a and -520c (477), as miR-106b is downregulated in AD (241). It should be noted though, that there is little overlap in the expression pattern of miRNAs in AD samples reported in the different studies. This might be attributed to the unstable nature of the miRNAs as demonstrated by Sethi et al. (549), or differences in the stage of the disease, or sampling sites. Thus more studies are required to confirm these findings.

#### 2. Parkinson's disease

Progressive neurodegeneration of dopaminergic neurons (DN) in the substantia nigra is the underlying cause of tremors, rigidity, and bradykinesia in Parkinson's disease (PD). Two different Dicer knockout models reveal a role for miRNAs in the function and survival of DN and dopaminoreceptive neurons. Dicer knockout in ES cells that coincides with the appearance of postmitotic DN (using the dopamine transporter promoter) results in complete loss of these neurons but not GABAnergic neurons (300). Similarly, knockout of Dicer in the postmitotic midbrain DN results in progressive loss of these neurons via programmed cell death and bradykinesia (300). One of the miRNAs that was suggested to be involved in the survival of DN is the muscle-enriched miR-133, which was also found in the midbrain of normal subjects but is absent in PD patients. The results show that miR-133b inhibits DN differentiation by targeting Pitx3; however, this finding disagrees with the loss of DN in the Dicer knockout. On the other hand, miR-133 has been shown to inhibit apoptosis in cardiac myocytes by targeting caspase-9 (679) and, thus, its loss in PD patients may trigger apoptosis of DN.

In contrast to those results, knocking out Dicer in postmitotic dopaminoreceptive neurons in the striatum and deep cortical layers (using dopamine receptor-1 promoter-driven Cre) did not result in neurodegeneration, but was associated with ataxia, reduced brain size due to smaller cells, astrogliosis, behavioral changes that impacted feeding habit, loss of weight, and premature death at 10–12 wk (128). α-Synuclein is also an underlying cause of DN dysfunction and degeneration in PD. Thus a reduction in miR-7, which targets its 3′-UTR, might underlie an increase in α-synculein protein, which forms the characteristic fibrillar aggregates in nigral PD (286). Notably, α-synculein is also induced by FGF10, which was identified as a risk factor at 8p21.3–22 with a SNP in the miR-433-binding site within its 3UTR (646).

#### 3. Huntington's disease

Huntington's disease (HD) is characterized by the gradual atrophy of the striatum that involves loss of neurons (reviewed in Ref. 225). Some of the underlying causes include dopmaine toxicity, apoptosis, and autophagy. The mechanism has been partly attributed to increased activity of the REST as it dissociates from the mutant huntingtin protein (719), which is known to suppress the transcription of neuronal genes. Interestingly, these genes include the brain-enriched miR-124a (114), which, as described above, is involved in the timining of neuronal differentiation. Conversely, miR-124 suppresses the expression of non-neuronal genes (114). HD is associated a decrease in mature miR-9/9*, miR-124, miR-29b, and upregulation of mature miR-132 (467), of which, at least, miR-124, miR-9, and miR-132 genes have RE1 binding sites in their vicinity (670). miR-9/9* is involved in a double negative feedback loop in which miR-9 targets and suppresses REST, while miR-9* targets and suppresses CoREST (467). These finding suggest that downregulation of miR-9 and miR-124, and upregulation of REST in HD, may be involved is suppressing any de novo differentiation of neural stem cells, with eventual atrophy of the striatum.

### C. miRNAs in Psychiatric Disorders

#### 1. Schizophrenia

Schizophrenia is a psychiatric disorder with a genetic predisposition that is also associated with differential expression of miRNAs (39, 482). One of the highest genetic risk factors known is a hemizygous microdeletion of the 22q11.2 locus, which is found in 1–2% of cases. To characterize the effects of this deletion, Stark et al. (579) generated a mouse model with an equivalent deletion that spans a 1.3-Mb region and encompasses 27 genes [*Df*(16)$A^{+/-}$ mice]. As predicted, this mouse exhibited some cognitive and behavioral changes that were reminiscent of schizophrenic traits. Most interesting, gene expression profiling revealed that several probe sets in close proximity to miRNA genes, including the miRNAs-containing gene (Mirg), were among the top scoring upregulated probes in the prefrontal cortex (PFC) and hippocampus. Notably, the Mirg is abundant in the adult brain and encompasses the largest known cluster of miRNAs, including miR-134, -136, -127, -154, -342, and -345, among several others (543). This finding led to the prediction that haploinsufficiency of *Dgcr8,* which lies within the 22q11.2 syntenic mouse locus, may be responsible for impaired pri-miRNA processing. In support, 59 miRNAs in the PFC and 30 in the hippocampus were downregulated. These included miR-185 and miR-134 that were present in the microdeletion. In accordance, the targets of these miRNAs were preferentially upregulated. As proof of a role for *Dgcr8* in schizo-

phrenia, heterzyogous *Dgcr8* knockout mice exhibited a phenotype similar to that of the *Df*(16)A$^{+/-}$ mice. In contrast, though, others have shown that in brain samples of schizophrenic patients, there is a generalized increase in miRNAs' expression that was associated with an increase in DGCR8 (38). Thus a more detailed sample assessment is necessary to determine the role of DGCR8 in schizophrenic patients.

Several SNPs are associated with this disease, such as those in the genes for miR-206, miR-198 (233), and miR-30e (682). In addition, by sequencing 59 miRNAs genes on the X-chromosome of male schizophrenic patients, Feng et al. (176) found eight ultra-rare SNPs in mature or precursor miRNA sequences. These included nucleotide variations in the mature sequence of let-7f-2, miR-188, -325, -660, -509–3, and -510, which altered the degree of their inhibition of target genes, and in the precursor of miR-18b, -505, and -502, which impaired their processing. SNPs are also found in the miRNA-binding sites in genes, such as that found in complexin 2 (CPLX2). Complexins 1–4 (CPLX1–4) are a family of presynaptic regulatory protein, of which CPLX2 was recently tested for its association with schizophreia, using a phenotype-based genetic association study in a relatively large cohort (26). This led to the identification of a C-to-T SNP in the 3′-UTR binding site for miR-498 seed sequence, which abrograted the corresponding miRNA's inhibitory effect on this gene. In concordance, this SNP was associated with an increase in the expression of the CPLX2 mRNA in peripheral blood mononuclear cells of patients.

### 2. Tourette's syndrome

Noteworthy, a G-to-A nucleotide variant (var321) was specifically found in 2 of 174 unrelated patients with Tourette's syndrome, in the binding site of miR-189 within the 3′-UTR of Slit and Trk-like (Slitrk1) (1, 460). The mutation enhances the repression of Slitrk1 by miR-189, as both molecules colocalize in the neocortex, hippocampus, thalamus, and cerebellum. Slitrk1 is a brain-restricted transmembrane protein with an extracellular region that has homology to Slit, an axonal growth factor and an intracellular domain with homology to trk neurotrophin receptors (17). The protein was shown to induce unipolar neurites in cultured neuronal cells (17). Moreover, a mouse model lacking Slitrk1 exhibits an anxiety-like behavior that is suggestive of a Tourette's syndrome trait (288).

## D. miRNAs in Brain Tumors

### 1. Gliomas

Gliomas are the most common form of adult brain tumors, of which glioblastoma multiforme is the most aggressive, grade IV glioma, and has received the most attention in miRNA studies. miR-21 is one of the most commonly upregulated miRNA in cancers, including glioblastoma, breast, ovarian, colorectal, pancreatic, lymphomas, lung, hepatocellular, prostate, head and neck, gastric, thyroid, cervical, and cholangiocarcinoma. It has an antiapoptotic function in glioblastoma cells, where its knockdown induces activation of caspase-3 and apoptosis (82). Interestingly, gene ontology analysis of miR-21 predicted targets revealed a significant bias in cell growth, proliferation, cell death, cancer, and cell cycle genes (469). With the use of this set of annotated genes, three interacting networks could be predicted that included genes from the transforming growth factor (TGF)-$\beta$, p53, and mitochondrial apoptotic pathways. In agreement, knockdown of miR-21 results in activation of these pathways, cell cycle arrest, and apoptosis, plausibly through its validated targets, which include heterogeneous nuclear ribonucleoprotein K (HNRK), p53-related TAp63 (469), and PDCD4 **(FIGURE 6)**, in glioblastoma cell lines (98). In addition, miR-21 is also involved in cell invasion and metastasis. It targets the metalloproteinase (MMP) inhibitors, reversion-inducing-cysteine-rich protein with kazal motifs (RECK) and tissue inhibitor of metalloproteinase-3 (TIMP3) and, thus, activates MMPs and facilitates cell motility and metastasis both in vitro and in vivo (192). While miR-21 has also been reported to target PTEN in other cell types, this has not been shown in gliobastomas. On the other hand, miR-26a is another PTEN-targeting miRNA that is upregulated in glioblastomas through amplification of it locus (257). It is plausible that both miRNAs collaborate to effect a more robust inhibition of PTEN and activation of AKT. While miR-21 is evenly upregulated in all glioma tumors, miR-221 is exceptionally higher in glioblastoma multiforme (grade IV) (116). miR-221 and miR-222, which are coexpressed, were found to target and inhibit the cell cycle inhibitor p27$^{kip1}$ in glioblastoma cells (206). Recently, the AKT pathway through inhibition of FOXO3a was also found to inhibit p27kip1 expression (555). Thus miRNAs may form an integrative network with other signaling pathways to potentiate their effects.

miR-21, miR-26a, and miR-221/222 function as oncomiRs by suppressing genes that negatively regulate cell growth, survival, and invasion. In contrast, miR-7, miR-34a, miR-124, miR-137, miR-146b, miR-15b, miR-128, and miR-326 function as tumor suppressors and are, thus, downregulated in glioblastomas. Downregulation of miR-7 complements the suppression of the AKT inhibitor PTEN by miR-26a, by directly derepressing the translation of epidermal growth factor receptor (EGFR) and insulin receptor substrate-1, both of which contribute to activation of the AKT pathway (295). In accordance, overexpressing miR-7 reduced cell survival and invasiveness of glioblastomas. Meanwhile, miR-34a (365) and miR-326 (294) are also downregulated and result in an increase in their common target Notch-1, which is known to enhance the expression of EGFR (496). In a negative feedback loop, Notch-1 was

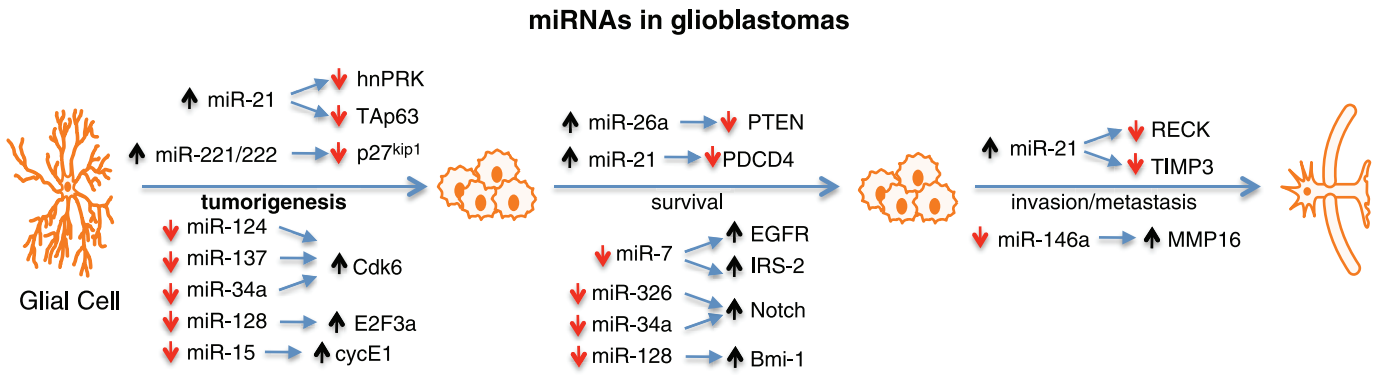


**FIGURE 6** A diagram showing miRNAs and their targets in glioblastomas. The diagram displays the different miRNAs and their targets that are involved in transformation, survival, and invasion/metastasis of glioblastomas. Upregulation or downregulation of a specific miRNA is represented by an upward (black) or a downward (red) arrow, respectively. The changes in the expression levels of a target gene inversely correlate with that of the targeting miRNA and are similarly represented by an up or down arrow. All listed targets have been validated. These include the following: heterogeneous nuclear ribonucleoprotein K (hnRPK), tumor protein p63 (TAp63), cyclin-dependent kinase 6 (Cdk6), E2F transcription factor 3 (E2F3a), phosphatase and tensin homolog (PTEN), programmed cell death 4 (PDCD4), epidermal growth factor receptor (EGFR), insulin receptor substrate 2 (IRS-2), BMI1 polycomb ring finger oncogene (Bmi-1), reversion-inducing-cysteine-rich protein with kazal motifs (RECK), tissue inhibitor of metalloproteinase 3 (TIMP3), and matrix metallopeptidase 16 (MMP16).

shown to inhibit miR-326 (294). Moreover, downregulation of miR-128 enhances the expression of its target Bmi-1 (208), which suppresses PTEN and increases AKT activity (570). Similarly, downregulation of miR-146b complements miR-21's suppression of MMP inhibitors, by relieving the inhibition of MMP16, and, thus, enhancing migration and invasion of glioblastoma cells (673). More direct effects on the cell cycle proteins are seen through downregulation of miR-15b, which targets cyclin E1 (672), miR-128, which targets E2F3a (710), and miR-124 and miR-137, which target cdk6 (562), augmenting the effect of miR-34a on the same target (365) **(FIGURE 6)**.

### 2. Medulloblastoma

Medulloblastoma (MB) is the most common form of childhood brain tumors, which may originate from granular cell progenitors (GCP) that fail to differentiate. Sonic Hedgehog (Shh) is a key regulator of cerebellar development, where it is responsible for the expansion of the GCP (623). miRNA, including miR-125b, miR-326, and miR-324–5p, enhance GCP differentiation by targeting the Hedgehog pathway regulator Smoothened (Smo), the latter also targeting the downstream transcription factor Gli (179). These progenitor cells are the most proliferating in the developed brain, where mutations in Shh pathway can result in MB. Indeed, downregulation of miR-125b, miR-326, and miR-325-p is observed in MB with high Shh signaling. In combination, these miRNAs are both necessary and sufficient for cell proliferation of MB cells. In addition, the miR-17~92a cluster was specifically upregulated in tumors with increased Shh activity (622). Overexpression of this cluster accelerated tumor formation and induced upregulation of Shh pathway mediators Math1 and Gli1. It is important to note, though, that it did not downregulate any of the targets reported in other cell types, including PTEN, p27kip, p130, or E2F-1, emphasizing the importance of the cell background in defining a miRNA's targets. In addition, the Notch receptor pathway also plays a role in MB where it is regulated by miR-199b-5p, which targets its effector HES1 (197). Lower levels of miR-199b-5p positively correlate with metastasis of MB.

## E. miRNAs in the Inner Ear

The mouse inner ear hair cells are particularly enriched in miR-182, -183, and -96 (miR-183 family), which are expressed early during the postnatal period and persist into adulthood (660). Conditional Dicer knockout in the otic placode at E8.5 demonstrates the importance of miRNA in inner ear development and, in particular, a role for miR-183 in the formation of the stereocilia of hair cells (573). Specific Dicer knockout in the hair cells furthers confirms the role of miRNAs in the formation of stereocilia (185). Likewise, studies in zebrafish show that knockdown of the miR-182, -183, and -96 results in a reduction in the number of inner ear hair cells (356). These data are suggestive of a role of the miR-183 family in hearing via regulating the proper development and number of the hair cells. Certainly, this was confirmed when two mutations in miR-96's seed sequence were found to segreggate with nonsyndromic hearing loss in two affected families (415). These mutations not only impaired miR-96's inhibition of its target genes but also compromised processing of its precursor. Moreover, one of the mutations converted miR-96's seed sequence into that of miR-514. Thus these mutants have potentially acquired

new targets, of which some were experimentally confirmed. Excitingly, this is the first pathogenic miRNA mutation to be confirmed in humans. Moreover, this finding was corroborated in a mouse model, in which a hemizygous *N*-ethyl-*N*-nitrosurea-induced mutation in miR-96 resulted in progressive hearing loss (354). This was associated with degeneration of all hair cells in the homozygous mice by 4–6 wk and the outer, but not inner, hair cells in the heterozygous mice. While the miR-96-binding site was enriched in the 3′-UTR of many genes that were upregulated in this model, the direct targets that mediate the effect of miR-96 remain to be identified.

## VII. MicroRNAs IN THE KIDNEY

### A. miRNAs in Nephrogenesis

Dicer or Drosha knockout in *Xenopus* (2- to 4-cell stage) results in gross malformation and various organ defects, including a reduction in the pronephric tubules, in addition to what appears to be delayed differentiation of the epithelia of the pronephric duct (5). Closer examination of the pronephros revealed that the proximal tubule marker $Na^+$/glucose cotransporter (SGLT1-K) was reduced, the intermediate tubule and part of the distal tubule were less convoluted, and the distal tubule was shorter. This implicates miRNAs in later versus earlier stages of pronephros development, including the timing of pronephric duct differentiation and tubule morphogenesis. Compared with other miRNAs expressed in the kidney, the miR-30 family was most restricted to, and highly abundant in, the pronephros. Thus, not surprisingly, when abrogated it induced a phenotype very similar to that of Dicer's ablation (5). Although miR-30 knockdown reduced proliferation, that function was not responsible for the phenotype. On the other hand, its direct inhibition of the LIM-class homeobox factor Xlim1 may be accountable, as its downregulation in the developing pronephros was delayed.

In a mouse model, podocyte-specific knockout of Dicer results in proteinuria within 2–3 wk, associated with glomerular hypertrophy and tubular dilatation, followed by death due to kidney failure (236, 245, 556). The histological findings include foot process effacement, accumulation of lipid droplets, and cytoplasmic vacuoles. Additionally, the basement membrane exhibits focal splitting, inclusions, and subepithelial projections. At the molecular level, the mutant podocytes had lower levels of the cytoskeletal proteins synaptopodin, ezrin, meosin, and podocalyxin. In addition, they had lower podocyte-specific nephrin, podocin, and CD2-associated protein (CD2AP) proteins, albeit levels of the Wilm's tumor (WT) transcription factor were normal. Aberrant proliferation and apoptosis are also observed at the later stages, which suggest that they are an effect rather than a cause of the phenotype. mRNAs profiling in the Dicer-deficient podocytes uncovered 190 genes that were upregulated whose 3′-UTRs were enriched in target sites for 16 miRNAs, which included the 4 miR-30 family members (556). Two of those targets are vimentin and HSP20 that may be involved in the underlying cytoskeletal changes observed in the mutant podocytes.

On the other hand, Dicer knockout in the ureteric bud derivatives results in unilateral or bilateral hydronephrosis in 47% of the mice, with occasional cystic tubules (476). Although the roles of specific miRNAs in the mammalian kidneys have not been identified yet, the Dicer knockout model proved their requirement for normal podocyte differentiation and ureteric development. In addition, the knockout of Dicer in *Xenopus* proved a role for miRNAs in the accurate timing of epithelial differentiation in the pronephric duct.

### B. miRNAs in Polycystic Kidney

Adult-onset autosomal dominant polycystic kidney disease (ADPKD) is characterized by formation of liver and kidney cysts. While the mechanism is not fully understood, it has been partly attributed to enhanced proliferation of the epithelium of renal and biliary ducts (35). As miRNAs play a major role in proliferation of cancer cells, they were predicted to similarly play a role in the pathogenesis of ADPKD. Differential display of cholangiocytes from a rat PKD model revealed downregulation of miR-15a; a similar finding was observed in the liver of ADPKD patients (342). This is consistent with other findings that implicate reduced miR-15 levels in the mechanisms underlying a wide range of cancers (24, 43, 53, 55, 66, 111). In colangiocytes, miR15a targets the cell cycle regulator Cdc25A and, thus, its downregualtion increases the protein (342). Moreover, knockdown of miR-15a in normal colangiocytes enhances their potential to form cysts in a three-dimensional culture system, but the role of Cdc25A in mediating this effect was not examined. It is not clear, however, whether miR-15 plays a role in renal cystogenesis, since differential profiling did not reveal any changes in its level (468). Otherwise, there is a plethora of miRNAs that are downregulated in PKD, whereas miR-21 is the only one that is upregulated. Although not specifically interrogated in this tissue, miR-21 is known to play a major role in cell survival and invasion during carcinogenesis and may, thus, play a similar role in kidney cystogenesis.

### C. miRNAs in Diabetic Nephropathy

Diabetic nephropathy is mainly due to the effect of hyperglycemia and cytokines on renal mesangial cells and is characterized by mesangial cell hypertrophy and apoptosis and excessive deposition of extracellular matrix proteins. To identify the role of miRNAs in this process, differential profiling was performed on mesangial cells cultured in high-

glucose medium (651). This revealed a high expression level of miR-377 that was upregulated in a mouse diabetic nephropathy model and via stimulation with TGF-$\beta$. Overexpression of this miRNA in mesangial cells results in an increase in fibronectin, most likely through an indirect mechanism. Indeed, miR-377 directly targets and inhibits p21 protein (Cdc42/Rac)-activated kinase 1 (PAK1) and superoxide dismutase (SOD1) in mesangial cells, where knockdown of either protein increases the production of fibronectin. On the other hand, TGF-$\beta$ plays a major role in the production of collagen in the diabetic kidney through inhibiting the expression of the E-box repressors, ZEB1 and ZEB2, which potentially suppress E-box-dependent expression of collagen (290). miR-192, which is induced by TGF-$\beta$, mediates its inhibition of ZEB2 via targeting that gene's 3′-UTR (290). Interestingly, suppression of ZEB2 also results in directly derepressing the expression of the gene encoding miR-216a and miR-217, both of which directly target PTEN, a negative regulator of the AKT pathway (289). This finding provides a unique mechanism for TGF-$\beta$-mediated activation of AKT. Indeed, TGF-$\beta$ stimulates AKT activity in a miR-21-PTEN-dependent fashion and increases phosphorylation of its downstream effectors, mTOR, FOXO3a, and GSK3$\beta$. This pathway, which is well established in mediating cell hypertrophy in various cells types, may play a role in the pathogenesis of diabetic nephropathy.

### D. miRNAs in Renal Cancer

Renal cell carcinoma (RCC) is the third most frequent urological cancer, of which clear cell carcinoma (CCC) constitutes ~70% and is associated with a high mortality rate compared with the less frequent chromophobe cell carcinoma (ChCC). A differential display revealed that miR-200c and miR-141 are preferentially downregulated in CCC (440). Both these miRNAs were found to target and inhibit the E-box repressor ZEB2, which is involved in endothelial-to-mesenchymal transition (EMT). This finding is common to many other cancer forms (2, 63, 215, 310, 313, 472) and is consistent with the fact that the milder form of RCC is not associated with downregulation of the miR-200 family (440). WT, on the other hand, has a unique pattern of gene expression that differentiates it from all forms of RCC (314). This pattern represents an E2F3 signature, a transcription factor frequently amplified in many forms of cancer, including WT. Similar to its effect in Burkitt's lymphoma (668), E2F3 induces upregulation of the miR-17~92 cluster.

## VIII. MicroRNAs IN THE LUNG

### A. miRNAs in Lung Development

The first indication that miRNAs were involved in lung development was provided by a knockout mouse model in which the Dicer gene was ablated in sonic hedgehog-expressing cells, which include lung epithelial cells (234). This disruption resulted in the cessation of lung branching at day 12.5, while the epithelial cells continued to proliferate and form large dilated distal ends of the existing branches. Although there was increased apoptosis in the mutant lungs, it was mostly observed at a later time point (E13.0), excluding it as a possible mechanism for the defective branching. The phenotype could be partly explained by upregulation of fibroblast growth factor 10 (FGF10) and its aberrant distribution within the mesenchyme. Since Dicer was deleted in the epithelium, whereas FGF10 is in the mesenchyme, its upregulation could be due to abnormal levels of other, secreted, regulatory factors. Specific miRNAs that play a role in lung development include miR-127. During lung development it is expressed in the mesenchyme and later in the epithelium, where its untimely overexpression disrupts branching of a fetal lung organ culture (41). Similarly, disrupting the normal expression pattern of miR-17~92, which is high during early development and low in the adult lung, via introducing a transgene in epithelial cells, results in delayed differentiation and epithelial hyperplasia at the expense of the alveolar cavity (390). Although retinoblastoma-like 2 (RBL2) was confirmed as target of miR-17–5p and is reduced in the transgenic model, its contribution to the phenotype has not been determined. This phenotype is consistent with the miR-17~92 knockout model, which is embryonic lethal and is associated with a hypoplastic lung but no branching or other obvious defects (633). Thus once more a miRNA (miR-17~92) regulates lung development by regulating the timing of epithelial cell development and exit from the cell cycle.

### B. miRNAs in Lung Cancer

Downregulation of let-7 has been one of the more consistent findings in lung cancer and, thus, the most investigated **(FIGURE 7)**. Furthermore, it is associated with poor prognosis after curative resection, independent of disease stage (595). Functionally, overexpression of let-7 inhibits growth of a lung adenocarcinoma cell line (323, 595). It also suppresses growth of tumor xenografts (166, 323) and primary tumors in a K-Ras$^{G12D}$ lung cancer model in vivo, when delivered intranasally via a lentivirus vector (166). Furthermore, let-7 does not only retard tumor growth, but also induces regression of established tumors through an apoptosis-independent mechanism (619). Conversely, inhibition of let-7 is sufficient for enhancing cell proliferation (282). Consistent with its targeting and suppression of K-Ras (283) and high mobility group AT-hook 2 (HMGA2) (346), let-7 was more effective in inhibiting growth of K-Ras mutant-positive versus -negative non-small cell lung cancer (NSCLC) cell lines, and its effect was reversed by reintroducing Ras, but less effectively by HMGA2 (323). The tumor suppressor effect of let-7 is further substantiated by its inhibition of the cell cycle proteins Cdk6 and cell division

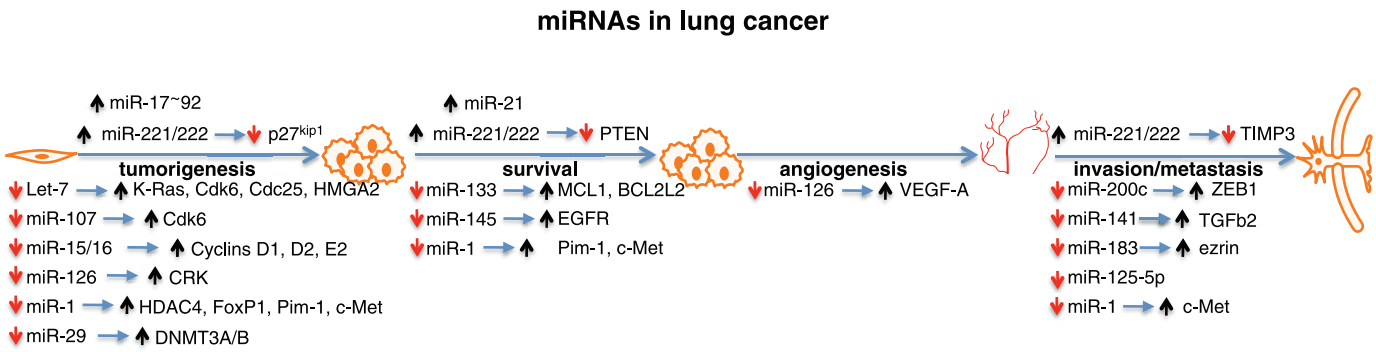


**FIGURE 7** A diagram showing miRNAs and their targets in lung cancer. The diagram displays the different miRNAs and their targets that are involved in transformation, survival, angiogenesis, and invasion/metastasis of lung cancer. Upregulation or downregulation of a specific miRNA is represented by an upward (black) or a downward (red) arrow, respectively. The changes in the expression levels of a target gene inversely correlate with that of the targeting miRNA and are similarly represented by an up or down arrow. All listed targets have been validated. These include the following: cell division cycle 25 (Cdc25), cyclin-dependent kinase 6 (Cdk6), high mobility group AT-hook 2 (HMGA2), K-Ras, v-crk sarcoma virus CT10 oncogene homolog (CRK), histone deacetylase 4 (HDAC4), forkhead box P1 (FoxP1), proviral integration site 2 (Pim-1), hepatocyte growth factor receptor (c-Met), DNA methyltransferase 3A/B (DNMT3A/B), myeloid cell leukemia sequence 1 (MCL1), Bcl2-like 2 (BCL2L2), epidermal growth factor receptor (EGFR), vascular endothelial growth factor A (VEGF-A), zinc finger E-box binding homeobox 1 (ZEB1), and transforming growth factor beta 2 (TGFb2).

cycle 25 (Cdc25) (282). Moreover, the existence of a single-nucleotide polymorphism within the let-7 target site in the 3′-UTR of K-Ras is associated with increased risk of NSCLC, underscoring its tumor suppressive role (106). It should be noted, however, that other miRNAs have essential and complementary roles in lung cancer. Specifically, miR-15/16, which is frequently deleted in leukemia, was also frequently reduced in lung cancer, where it directly influences the cell cycle by targeting cyclin D1 (cycD1), cyclin D2 (cycD2), and cyclin E1 (cycE1) (24). However, since these cyclins function with their kinase partners to phosphorylate and inhibit RB, the effect of miR-15/16 on proliferation is not observed in cancer cells deficient in RB. Additional tumor suppressors that are downregulated in lung cancer include miR-126 (126, 375), miR-107, and miR-185 (594). While miR-126 was found to target v-crk sarcoma virus CT10 oncogene homolog (CRK) and vascular endothelial growth factor A (VEGF-A), miR-107 targets Cdk6. On the other hand, downregulation of miR-29 contributes to tumorgenesis by derepressing the methyltransferase genes DNA (cytosine-5-)-methyltransferase 3 alpha (DNMT3A) and 3B, which invoke aberrant methylation and gene silencing (167).

Interestingly, the two muscle-enriched miRNAs, miR-1 and miR-133b, which are minimally expressed in normal bronchial epithelium, were found dramatically reduced in lung tumors (125, 443). Reexpressing miR-1 in cancer cell lines suppressed proliferation, migration, and tumorgenicity (443). It also induced apoptosis in response to anticancer agents. Some of miR-1's targets that were upregulated in this context included hepatocyte growth factor receptor (c-Met), proviral integration site 2 (Pim-1), forkhead box P1 (FoxP1), and HDAC4. In particular, c-Met has been previously implicated in lung tumorgenesis (495, 519, 578). In addition, Pim-1 has proliferative and prosurvival functions (677), HDAC4 inhibits the cell cycle inhibitor p21 (434, 665), while FoxP1's role is dependent on the type of cancer (311) and remains to be investigated in lung cancer. On the other hand, miR-133 overexpression was mainly shown to induce apoptosis in the presence of anticancer drugs, plausibly by targeting the established the prosurvival genes Bcl2-like 2 (BCL2L2) and myeloid cell leukemia sequence 1 (BCL2-related) (MLC1) (125).

In contrast to miRNAs that are downregulated, several were upregulated and are involved in lung cancer cell proliferation, survival, and resistance to chemotherapy **(FIGURE 7)**. This includes the miR-17~92 cluster that is increased in cancer, sometimes through gene amplification, and is involved in enhancing cell proliferation (238). In addition, its abrogation by antisense knockdown proved that it has an antiapoptotic function that is mostly mediated through the miR-17~5p and miR-20a members (409). However, its targets in this particular tissue have yet to be identified. On the other hand, c-Met via AP-1 induces upregulation of miR-221/222, which increases lung cancer resistance to the major extrinsic apoptotic pathway, stimulated by TNF-$\alpha$-related apoptosis inducing-ligand (TRAIL) (195, 196). This is accomplished by its direct targeting of PTEN, AKT's negative modulator, which has been previously shown to be responsible for resistance to TRAIL through preventing BID cleavage (447). Moreover, miR-221/222 targets TIMP3, a tumor suppressor gene and a negative regulator of cell invasion that is frequently methylated in cancer cells (20). miR-221/222 expressing cells also have an advantage in cell proliferation that could be attributed to the direct suppression of $p27^{kip}$ (196). In addition, miR-21 plays an antiapoptotic role in

this context, where it is induced by activated EGFR (542), which itself is induced as a result of a reduction in miR-145 (107). In contrast to the above listed antiapoptotic miRNAs, p53 induces apoptosis via upregulation of miR-34a, following its reactivation by PRIMA-1 (150) [a small molecule that restores mutant p53's transcriptional activity and apoptotic effects in lung cancer cell lines (64)].

In addition to regulating proliferation and viability of lung cancer cells, miRNAs are also involved in migration and metastasis **(FIGURE 7)**. One of the established mechanisms in cancer cell metastasis is the EMT of cells, which is associated with upregulation of vimentin and downregulation of cell border uvomorulin, thus increasing cell motility and invasiveness (21). EMT could be stimulated by TGF-$\beta$, TNF-$\alpha$, or HGF, which induce upregulation of the zinc-finger E-box binding homeobox (ZEB) protein that suppresses E-cadherin. The miR-200 family plays a major role in metastasis of lung, colon, and breast cancers. Using K-Ras/p53 double mutant cells that develop metastatic lung adenocarcinoma, Gibbons et al. (205) found that EMT required downregulation of the miR-200 family. In specific, miR-141 and miR-200c were >90% reduced in metastasized cells relative to the tumor of origin. Consequently, overexpression of miR-200b dramatically reduced TGF-$\beta$-induced EMT via suppression of its target ZEB1, but not ZEB2, and rescued the downregulation of E-cadherin. In contrast, stimulation of lung cancer cells with the tumor-promoting EGF did not significantly reduce miR-200c but did induce ~50% downregulation of miR-125–5p, as well as several members of the let-7 family. Furthermore, ectopic expression of miR-125–5p inhibited cell migration and invasion, but its targets here remain unidentified (645). Another miRNA that is involved in metastsis of lung cancer is miR-183. It is preferentially underexpressed in metastatic versus nonmetastatic lung cancer cells of the same origin and targeted the cytoskeletal protein Ezrin (644), which plays an established role in cell invasion and metastasis.

## IX. MicroRNAs IN THE LIVER

### A. miRNAs in Liver Development and Function

Consistent with a more subtle regulatory function, Dicer deletion did not affect early liver development even though the liver-specific miR-122 was almost completely lost for up to 3 wk postnatally (544). However, 3-wk-old livers were paler than normal and exhibited an increase in proliferation and apoptosis. In addition, cells had more lipid droplets but lacked glycogen granules, indicative of metabolic abnormalities. At the molecular levels, markers of terminally differentiated hepatocytes were normal in Dicer-deficient cells, but were accompanied by reiteration of the fetal gene program. Eventually, most Dicer-negative hepatocytes underwent apoptosis and were replaced by normal cells within 6–12 wk. Interestingly, though, the few that did survive contributed to high frequency of hepatocellular carcinoma in 6-mo-old mice.

Because the phenotype of the Dicer knockout mice was gradually reversed, the effect of miRNAs, including miR-122, on the adult liver function was not fully evaluated. Another study, using antisense knockdown of miR-122 in the adult liver, confirms its essential role in lipid metabolism (165). This was demonstrated by lower plasma cholesterol (26–28% lower) and triglyecride levels. Indeed, ex vivo experiments proved that the rate of sterol and fatty acid synthesis was decreased, while fatty acid oxidation was increased approximately twofold. This phenotype could be partly explained by a reduction in acetyl-coenzyme A carboxylase 2 (ACC2) and stearoyl-coenzyme A desaturase 1 (SCD1), and an increase in phosphorylated AMP-activated kinase alpha 1 subunit (AMPKa1), although miR-122 direct targets that elicit these effects were not identified in this study. In a third study, Elmen et al. (162) showed that antisense knockdown of miR-122 in vivo induced upregulation of some of its predicted targets that include aldolase A, branched-chain $\alpha$-ketoacid dehydrogenase kinase, Cd320 antigen, putative very-low-density lipoprotein receptor, and N-myc downstream regulated gene 3 (162). In addition, miR-122 is also responsible for almost complete suppression of the cationic amino acid transporter in the adult liver (84).

### B. miRNAs in Hepatitis

Hepatitis C virus (HCV) replicates specifically in hepatocytes, indicating the requirement for liver-specific factors. With the advent of miRNAs, it was also noted that HCV only replicates in the hepatoma cell lines that have detectable miR-122 (Huh7) but not those that are deficient in this miRNA (HepG2) (284). Depletion of miR-122 in Huh7 cells reduced the viral RNA replicon by ~80%, while increasing miR-122 increased its production, suggesting a role for miR-122 in HCV replication. Moreover, ectopic expression of miR-122 in human embryonic kidney HEK-293 cells facilitated HCV replication (83). Interestingly, two putative miR-122 targeting sites were discovered in the RNA genome of HCV; two in the 5′ noncoding region (NCR) between the stem-loops I and II and one in the 3′ NCR, which were highly conserved among six HCV genotypes (244, 284). Mutation analysis revealed that only the former was necessary for miR-122-dependent viral replication, which appeared to require direct interaction with miR-122. This interaction, however, had no effect on the stability of the viral RNA, whereas reports on its role in gene translation are controversial (244, 284). While Jopling et al. (284) noted no effect on viral translation, Henke et al. (244) report that miR-122 enhances HCV replicon translation by stimulating the association of the small ribosomal subunit

with the viral replicon at the initiation stages. Consistent with the latter observation is the fact that the 5′ NCR regulates translation through stem-loop II-IV, which is the internal ribosome entry site (563) and where proximal binding of miR-122 has been shown to induce an "open" conformation that may favor translation (142). These findings suggest that miR-122 could serve as a major antiviral therapeutic target for treatment of hepatitis C. Indeed, a recent study shows that antisense antagonism of miR-122 in primates resulted in efficient reduction in serum (2.6 logs) and liver (2.3 logs) HCV, with no evidence of viral resistance or side effects (331). In addition to downregulation of miR-122, other antiviral miRNAs are induced by IFN-$\beta$ in Huh7 cells that include miR-1, miR-30, miR-128, miR-196, miR-296, miR-351, miR-431, and miR-448, all with HCV-complementary seed sequences that could contribute to a robust anti-HCV effect (478). Notably, though, in chronic hepatitis C (HC) patients, miR-122 levels did not correlate with HCV RNA levels and did not change in response to interferon treatment (529). However, the status of miR-122 in acute cases of HC remains to be examined.

### C. miRNAs in Hepatocellular Carcinoma

Downregulation of the liver-enriched miR-122 has been a consistent finding in both rodent and human hepatocellular carcinoma (HCC). This finding was observed in ~50% of primary human tumors (324) as it appeared to correlate with poor prognosis, larger tumor size, c-Met positivity, and loss of the hepatocyte differentiation (123). Moreover, overexpression of miR-122 suppressed HCC growth and migration both in vitro and in vivo, and vice versa (23, 123). Some of the targets that are likely to mediate its effects and whose expression inversely correlate with that of miR-122 in tumors include disintegrin and metalloprotease 17 (ADAM17) (621), serum response factor (SRF), and IGF-IR (23). Another miR-122 validated target that is significant in hepatoceullar carcinogenesis is cyclin G1 (212). In accordance, overexpression of miR-122 in hepatcelullar carcinoma cells suppressed cyclin G1 and enhanced phosphorylation of p53 (183). Indeed, cyclin G1 null mice have a higher content of p53 and lower incidence of hepatic tumors (273). Moreover, overexpression of miR-122 or knockdown of cyclin G1 enhanced E-cadherin expression and reduced cell invasion and increased apoptosis. However, whether cyclin G1 is necessary or sufficient for mediating miR-122's effects remains unexamined. In addition, a proapoptotic function of miR-122 is possibly mediated by its direct targeting of Bcl-w (a Bcl-2 family member) (368).

Other growth suppressor miRNAs include miR-195, which is depressed in HCC and where its replenishment inhibits growth, possibly by targeting cyclin D1, Cdk6, and E2F3 (681); and miR-1, by targeting FoxP1 and HDAC4 (132). Downregulated miRNAs in HCC that were shown to specifically inhibit cell invasion and migration include miR-34a (357), miR-23b (523), and miR-1 (132), which all target c-Met; and miR-101, which inhibits AP-1-dependent transcription through targeting v-*fos* (360). In addition, other miRNAs were identified as mediators of apoptosis and are also reduced in HCC, these include miR-101, which targets the antiapoptotic protein Mcl-1 (583), and miR-233, a suppressor of stathmin (667), a microtubule-destabilizing and tumor-promoting protein (129, 204, 429). Considering the preponderance of miRNAs that are downregulated in HCC, Kato et al. (315) examined the efficacy of miRNA replacement therapy for its treatment. For that purpose, they selected miR-26a, which they found downregulated in a mouse model of Myc-induced HCC, as well as human HCC samples. Another criterion they sought was a miRNA, normally highly expressed in all organs, that was reduced only in the tumor, which they predicted would be generally well tolerated if modestly overexpressed. In this case, they delivered miR-26a intravenously via an adenoassociated virus. miR-26a, which they found to target cyclins D2 and E2, impressively reduced tumor burden in the liver. This effect was a result of reduced proliferation and increased apoptosis of the tumor cell.

Furthermore, PPAR$\alpha$ plays a role in HCC by activating a miRNA-dependent pathway that is initiated by downregulation of the tumor suppressor let-7 and an increase in its target c-*myc* (550). This, in turn, induces upregulation of the oncogenic miR-17~92 cluster (550). The latter was found upregulated in 100% of human HCC and is involved in promoting proliferation and anchorage-independent growth of cancer cells (115). Although the targets of miR-17~92 have not been specifically identified in HCC, that of its paralog, miR-106b~25, were further investigated. Data show that in HCC miR-25 targets the proapoptotic protein Bim, while miR-106b targets E2F1 (366). It was predicted that suppression of E2F1 might be necessary for regulating any excessive increase of the protein that may elicit an adverse apoptotic function. Other miRNAs with oncogenic functions (oncomirs) in HCC include the following: miR-21, which suppresses PTEN and is associated with an increase in phospho-AKT, phospho-focal adhesion kinase (p-FAK), and metalloproteinase 2 (MMP2) (417); miR-221, which inhibits the cell cycle inhibitors p27 and p57 (183), and BH3-only protein Bmf (213); miR-155, which targets c/EBP$\beta$ (643); and miR-145, which enhances cell invasion by targeting fibronectin type III domain containing 3B (709).

## X. MicroRNAs IN THE PANCREAS

### A. miRNAs in Pancreas Development and Function

Conditional knockout of Dicer in the pancreas uncovers the requirement of miRNAs during its development in mice

(396). The development of all the endocrine cell lineages, especially β-cells, was dramatically impaired in this model, which was lethal before P3. The phenotype was mostly due to a reduction in progenitor cells as a result of an increase in the notch-signaling target Hes1 and cellular apoptosis rather than a reduction in cell proliferation, as evidenced by reduced neurogenin3-positive progenitors. This is also supported by the fact that postdevelopment β cell-specific deletion of Dicer had no effect on β-cell proliferation or maintenance. On the other hand, a generalized Dicer-hypomorphic knockout model with only ~20% residual dicer in all tissues, displayed abnormalities in the adult pancreas in the form of irregular distribution of islet cells, an increase in the number of ductal insulin+/glucagon+/Pdx-1 epithelial cells, and abnormal multinucleated cells, which argued that miRNAs might be regulating progenitor cell proliferation and differentiation in this organ (431).

Cloning of miRNAs from pancreatic islet cells revealed the expression of the tissue-enriched miR-375 (492). In vivo promoter expression studies revealed that it is specifically transcribed in α-, β-, and δ-islet cells of the adult pancreas via the helix-loop-helix Id2 and Id3 factors (19). miR-375's function proved inhibitory to glucose-induced insulin secretion by suppressing exocytosis, but otherwise had no effect on insulin synthesis, $Ca^{2+}$ signaling, availability of granules, or submembrane actin network (492). One of its identified targets includes V1/myotrophin, whose knockdown mimicked miR-375's effect in β-cells. A second study, while confirming that miR-375 inhibits insulin secretion, shows that it does so through PDK1-dependent inhibition of insulin synthesis (156). Accordingly, glucose enhances insulin expression via inducing downregulation of miR-375 and upregulation of its target PDK1. However, glucose's effect is not limited to miR-375; it also increases the expression of several miRNAs including miR-30d, which plays a role in enhancing the expression of insulin in a β-cell line (597). Conversely, miR-9, a brain-enriched miRNA, is expressed in islet cells and inhibits glucose-induced insulin secretion through suppression of Onecut-2, which, in turn, enhances the expression of the negative regulator granuphilin/Slp4 (488). Thus synthesis and secretion of insulin are a function of at least three miRNA (miR-375, miR-30d, and miR-9) that might operate as a molecular switch during hyperglycemia.

Targeted deletion of miR-375 in a mouse model showed that it is also required for regulation of β to α cell ratio during development (493). This phenotype was evident at 3 wk of age, when the pancreas was found to have 38% less β-cells versus 70% more α-cells. The increase in α-cells was responsible for an increase in plasma glucagon, hepatic gluconeogenesis, and hyperglycemia in these mice. In contrast, the obese mouse model exhibits an increase in β-cells due to insulin resistance, which is associated with a 30% increase in miR-375. Cross-breeding the two models resulted in decreasing the levels of β-cells in the obese mouse, reducing insulin production, and exacerbating the diabetic phenotype. At least 10 of miR-375's targets were upregulated in pancreatic islets from the knockout model. Those included the following: caveolin1 (Cav1), inhibitor of DNA binding 3 (Id3), SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 2 (Smarca2), Ras-dexamethasone-induced-1 (Rasd1), regulator of G protein signaling 16 (Rgs16), eukaryotic elongation factor 1 epsilon 1 (Eef1e1), apoptosis-inducing factor, mitochondrion-associated 1 (Aifm1), cell adhesion molecule 1 (Cadm1), HuD antigen (HuD), and complement component 1 q subcomponent binding protein (C1qbp). This set of genes is known to negatively regulate cell growth and proliferation that partly explains the reduction in β-cells. In addition to miR-375, other islet-enriched miRNAs include miR-7 (59, 119, 278), whose function remains to be explored.

## B. miRNAs in Pancreatic Cancer

Expression profiling of miRNAs in pancreatic cancer (PaCa) versus chronic pancreatitis identified upregulation of 15 and downregulation of 8 miRNAs that accurately classified 93% of the samples (47). Moreover, increased expression of miR-196a and miR-219 emerged as predictors of poor survival rate. miR-196a/b's role was further emphasized as it was strictly detected in cancer tissue and cell lines but not in normal tissue or in pancreatitis (591). Other notable miRNAs that were identified in this screen included miR-21, miR-221, and miR-155 that were upregulated, and miR-375, which was downregulated (47). miR-21 is upregulated in ~79% of pancreatic tumors, and its levels are predictive of survival in patients that are negative for lymph node metastasis; however, it does not correlate with tumor size, differentiation, or stage (143). Knocking down miR-21 in PaCa cells reduced proliferation and invasion and sensitized them to chemotherapeutic agents as two of its targets, PTEN and reversion-inducing cysteine rich protein with Kazal motifs (RECK), were upregulated (432, 471). A similar effect is exerted by miR-221 via targeting the cell cycle inhibitor $p27^{kip1}$ (471). In addition, miR-21 and miR-155 are two of the most highly expressed in early noninvasive intraductal papillary mucinous neoplasms of the pancreas (228), the latter targeting tumor protein p53-induced target gene (TP53INP1) and inhibiting p53-induced apoptosis (207).

Consistent with other forms of cancer, the miR-200 family of miRNAs is downregulated, promoting EMT and cell invasion (63, 659). A double negative feedback loop is created as ZEB1 directly suppresses the expression of miR-200c and miR-141, while miR-200c represses ZEB1 expression and miR-141 represses TGFβ2, an inducer of EMT. In addition to the enhanced invasiveness associated with EMT, recent studies show that cells that undergo EMT also

acquire stem cell markers (403). Accordingly, ZEB1-deficient tumors were not only impaired in their metastatic capacity, but were also smaller in size, which supports the idea of reduced numbers of cancer stem cells (659). In agreement, ZEB1 knockdown in MiaPaCa2 reduced the number of stem cells and their sphere-forming capacity and resistance to chemotherapy. This was confirmed by lower expression of stem cell factors including Sox2, bmi1, and p63. In addition to the miR-200 cluster, ZEB1 was shown to directly suppress miR-203 and had putative binding sites in miR-183's promoter region. Indeed, overexpression of either in PaCa cells reduced the number of cancer stem cells. miR-200c, miR-203, and miR-183 all targeted Bmi1, a protein known for its function in stem cell renewal (470), while miR-200c additionally targets Sox2 and klf4. Furthermore, overexpression of any these miRNAs reproduced the effects of ZEB1 knockdown in cancer cells. Similarly, miR-34a suppresses cancer stem cell proliferation and is usually downergulated, deleted (85), or methylated in PaCa (380). Overexpression of miR-34 in a pancreatic cancer cell line reduced the number of cancer stem cells and, thus, its capacity for tumor formation in nude mice (274). This was associated with downregulation of its targets Bcl-2, notch-1, and notch-2 proteins, apoptosis, cell cycle arrest, and reduced invasion (274). Thus these results reveal an intricate network between ZEB1, miR-200, miR-203, miR-183, and miR-34a, that promotes EMT and stemness in PaCa. However, EMT is not the only mechanism that promotes metastasis, as miR-10a was found to promote metastasis through suppression of HOXB1 and HOXB3 (658).

## XI. MicroRNAs IN THE SKIN

### A. miRNAs in Skin Morphogenesis

Like other major organs, the skin harbors at least one predominant, tissue-enriched miRNA, namely, miR-203, which is highly conserved and specifically enriched in keratinocyte (571). miR-203 is upregulated in differentiating keratinocytes (348, 691) and is, thus, enriched in the suprabasal epithelial cells and in differentiated epidermal cells, while being absent in the proliferating basal layer in both mouse (348) and human (657) skin. Ectopic expression of miR-203 in the basal layer of stratified epithelium results in flattened basal cells and only a single layer of suprabasal cells and, thus, thin skin, attributed to premature differentiation and depletion of the basal progenitor cells (348). In accordance, these mice die shortly after birth from apparent dehydration. Conversely, knockdown of miR-203 using locally applied antisense constructs, delayed cell cycle exit of the suprabasal epithelial cells. In agreement, tissue-specific knockout of *Dicer1* led to thickening of the epidermis. The levels of miR-203 inversely correlated with that of the transcription factor p63, which is known for maintaining "stemness" of the progenitor cells (547). Indeed, p63 proved to be a direct target of miR-203 (348, 691). In accordance, the phenotype of the miR-203 transgenic mouse may be ascribed to suppression of p63, as it recapitulates the phenotype of the p63 null mice (547). The data suggest that miR-203 regulates the timing of cell cycle exit and differentiation of skin stem cells.

### B. miRNAs in Psoriasis

Psoriasis is a chronic inflammatory skin disease in which both the keratinocyte and infiltrating immune cells are involved in the formation of erythematous scaling plaques (336). miR-203 and miR-146a are specifically upregulated in cases of psoriasis, but not in eczema (571). This is in contrast to miR-21, which is upregulated in both lesions. Conversely, miR-125b is downregulated in both. Moreover, the cell-type distribution of these molecules is significantly different. Whereas miR-203 is restricted to keratinocytes, miR-146a is virtually undetectable in these cells or in dermal fibroblasts. On the other hand, miR-146a is relatively high in the infiltrating immune cells, consistent with its function in immune reactions, while miR-21 is generally ubiquitous.

In psoriasis, keratinocytes' proliferation is enhanced as their differentiation is retarded (382), rendering it hard to reconcile the function of miR-203 in psoriasis with its role in skin morphogenesis. The data suggest that miR-203 may have different targets in normal versus psoriatic keratinocytes, where it exists in supernormal levels. Surely, only in psoriasis, miR-203 targets SOCS-3, which is a negative regulator in cytokine signaling pathways and keratinocyte proliferation (372) and is downregulated in the psoriatic lesions (571).

### C. miRNAs in Malignant Melanoma

miRNA genes are frequently amplified or deleted in malignant melanoma (MM), breast cancer, and ovarian cancer (705). Among those frequently amplified in melanoma include miR-182 and miR-15/16, and those deleted include Let-7a/b. High levels of miR-15b were shown to correlate with poor recurrence-free and overall survival, possibly through promoting tumor survival (530). Meanwhile, miR-182 is found in the chromosomal region 7q31–34 that is frequently amplified and is, accordingly, upregulated in MM samples (541). In particular, its expression correlates with metastasis of human MM. It was also proven to enhance migration and survival of melanoma cells both in vitro and in a mouse model through targeting microphthalmia-associated transcription factor-M (MITF-M) and FOXO3. MITF is a regulator of melanocyte growth and differentiation and is frequently downregulated in melanomas (546). It is cleaved by caspases, rendering the COOH-terminal cleavage product proapoptotic (332). Promyelocytic leukemia zinc finger (PLZF) is another transcription

factor that is reduced in MM, unblocking the transcription of miR-221/222 (174). This miRNA suppresses $p27^{kip1}$, c-kit receptor, and its downstream effector MITF, thus enhancing cell proliferation and promoting dedifferentiation. In contrast to the aforementioned tumor promoting miRNAs, Let-7a is a tumor suppressor that is frequently downregulated in cancer (705). Let-7a inhibits MM cell migration, which could be explained by its suppression of β3 integrin (435), while Let-7b inhibits cell proliferation via targeting cyc D1, D3, and Cdk4 (538). The former is also a target of miR-193b, which is similarly downregulated in MM (90).

## XII. MicroRNAs IN COLON CANCER

Sequencing of miRNAs in colorectal cancer (CRC) revealed consistent downregulation of mature miR-145/143 but not their stem-loop precursor, indicating a posttranscriptional mechanism in the regulation of this cluster (424) **(FIGURE 8)**. miR-145/143, which are widely expressed in all normal tissues examined, were virtually undetectable in cancer cell lines, including colon, prostate, neuroblastoma, cervical, hepatic, and leukemia (8). Ectopically expressing either miRNA in colon cancer cell lines inhibited their growth (8, 554) and was associated with downregulation of miR-143's predicted target, Erk5 (8). miR-145 has been confirmed to target insulin receptor substrate 1 (IRS-1) (554). This adaptor protein mediates signaling of insulin and IGF receptors to their downstream AKT and Erk1/2 signaling pathways, while polymorphism of its gene is associated with a 70% increased risk of colon cancer (566). Thus derepression of IRS-1 by downregulation of miR-145 is one mechanism that enhances the expression of IRS-1 in cancer cells. Furthermore, miR-145 targets IGF-1 receptor, which emphasizes its role in regulating this pathway (325). However, while a miR-145-resistant IRS-1 gene was sufficient for reversing miR-145-induced growth inhibition of colon cancer cells, IGF-1R was not. The results are supported by the fact that miR-145 failed to inhibit the growth of a mammary cancer cell line that does not express IRS-1 (325). miR-143, on the other hand, was shown to target the protooncogene K-Ras (95) and DNA methyltransferase 3A (DNMT3A) (449), which is linked to hypermethylation and silencing of genes by the polycomb groups during carcinogenesis (276). K-Ras is also a validated target of let-7, which is also downregulated in 30% of CRC cases (7). It should be noted here that another study has identified miR-145 as an oncomiR rather than a tumor suppressor in metastatic colon cancer cells (16). However, this finding is hard to reconcile with the fact that miR-145 is consistently downregulated in primary tumors and most cell lines and that its levels inversely correlate with tumor size (565).

Gene silencing by CpG island hypermethylation is a common feature of cancer cells and is mainly achieved by the DNA methyltransferase (DNMT) family of proteins that are upregulated in cancer cells (reviewed in Ref. 158). Knockout of DNMT1 and DNMT3b in colon cancer HCT-116 cell line results in the upregulation of 18 miRNAs, 5 of which are embedded in CpG islands (393). In particular, the CpG islands harboring the three miR-124a genes are selectively hypermethylated in the cancer cells. Cdk6 was identified as a target of miR-124 in CRC, where its expression positively correlated with DNA hypermethylation and negatively with miR-124a levels. Another gene that is silenced in CRC tumors and cell lines by GpC hypermethylation is miR-34a/b/c (380, 617). Notably, transcription of the miR-34a gene is also dependent on p53 and is accordingly depressed in cancer cells lacking p53 (85, 118, 239, 503, 599). Moreover, a p53 enhancer that is in close proximity to the 5′-end of the miR-34a primary transcript is located in a CpG island (503). In support, hypermethylation of the gene

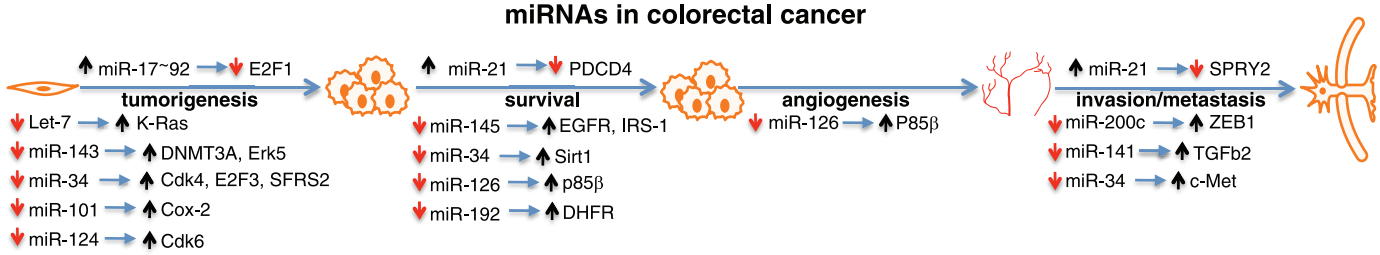


**FIGURE 8** A diagram showing miRNAs and their targets in colorectal carcinoma. The diagram displays the different miRNAs and their targets that are involved in transformation, survival, angiogenesis, and invasion/metastasis of colorectal cancer. Upregulation or downregulation of a specific miRNA is represented by an upward (black) or a downward (red) arrow, respectively. The changes in the expression levels of a target gene inversely correlate with that of the targeting miRNA and are similarly represented by an up or down arrow. All listed targets have been validated. These include the following: E2F transcription factor 1 (E2F1), K-Ras, DNA methyltransferase 3A (DNMT3A), mitogen-activated protein kinase 7 (Erk5), cyclin-dependent kinase 4 (Cdk4), E2F transcription factor 3 (E2F3), splicing factor, arginine/serine-rich 2 (SFRS2), Cox-2, cyclin-dependent kinase 6 (Cdk6), epidermal growth factor receptor (EGFR), insulin receptor substrate 1 (IRS-1), Sirtuin 1 (Sirt1), phosphoinositide-3-kinase, regulatory subunit 2 (beta) (p85β), dihydrofolate reductase (DHFR), zinc finger E-box binding homeobox 1 (ZEB1), transforming growth factor beta 2 (TGFβ2), and hepatocyte growth factor receptor (c-Met).

abrogated its activation by p53 (380). Thus hypermethylation and loss of p53 in cancer cell ensure silencing of miR-34, which underscores its tumor suppressive function. Some of its predicted targets that have been validated in CRC, include Cdk4, c-MET, splicing factor, arginine/serine-rich 2 (SFRS2) (617), and E2F3 (603). Also, by targeting and suppressing Sirt1, it increases acetylation and activation of p53, and thereby induces apoptosis in p53-positive CRC cells (683). In accordance, overexpression of miR-34 inhibits tumor growth in vivo (603). Likewise, a p53-binding site was identified in the promoter of miR-192 (569). Similar to miR-34, its expression positively correlates with that of p53, and it inhibits cell proliferation in a p53-dependent fashion, albeit to a lesser extent than that offered by miR-34 (58, 569). It was also shown to increase sensitivity to chemotherapeutic agent, plausibly by targeting dihydrofolate reductase (DHFR) (569).

Expression profiling of tumors identified 37 miRNAs that are differentially expressed in CRC (536). Specifically, high levels of miR-21 correlated with poor survival and resistance to therapy. miR-21 was also the only miRNA that was consistently upregulated in all solid tumors examined (639). Among the miR-21 targets that were validated in CRC is the tumor suppressor programmed cell death 4 (PDCD4) (18). This protein is known to interact with eIF4A and eIF4G and inhibit protein translation (687, 701), a mechanism which may explain its inhibition of neoplastic transformation (112). In addition, knockdown of miR-21 reduces invasion, extravasation, and metastasis of CRC cells (18), plausibly via enhancing the expression of its target Sprouty 2 (534), a known inhibitor of cell migration (337, 693) (703). Another mechanism that initiates cancer cell metastasis is EMT. It is induced by TGF-$\beta$, TNF-$\alpha$, or HGF and is associated with an increase of ZEB, which suppresses the expression of basement membrane proteins (574) and, thus, enhances metastasis in CRC (575). ZEB1 also suppresses the transcription of four miRNAs in CRC and breast cancer cells lines that include three members of the miR-200 family (miR-141, -200b, -200c) (63). Conversely, miR-141 and miR-200c inhibit EMT by directly targeting TGF-$\beta$2 and ZEB1 and function as a switch for EMT **(FIGURE 8)**.

Finally, it is worth noting that cyclooxygenase-2 (Cox-2), which is expressed in 86% of colorectal adenocarcinomas and 43% of adenomas (151) and plays a critical role in tumor formation (553), is also subject to regulation by miRNAs. Specifically, miR-101 is downregulated in CRC and directly targets and inhibits Cox-2 translation (582). Additionally, downregulation of miR-126 may indirectly participate in the regulation of Cox-2 via increasing the expression of p85$\beta$ subunit of the PI3-kinase and enhancing the activity of AKT (222), a positive regulator of Cox-2 (551). On the other hand, the role of p85$\beta$ in angiogenesis (700) suggests that downregulation of miR-126 in CRC may also participate in that process **(FIGURE 8)**.

## XIII. MicroRNAs IN BREAST CANCER

### 1. Tumor initiation (Figure 9)

Investigations of the tumorigenic potentially of mammary cancer cells led to the finding that only a minor subpopulation, with stem cell characteristic, had tumor initiating capacity (9). These breast cancer stem cells (BCSC) are characterized by a $CD44^+CD24^-$/lineage$^-$ phenotype that is utilized as a selection marker. miRNAs profiling of this subpopulation revealed reduced levels of let-7, which was gradually reversed upon their adherence and differentiation in culture (695). Accordingly, let-7 proved to be a negative regulator of the self-renewal, tumorigenic, and metastatic capacity of BCSC. Its expression inversely correlates with the expression of two of its targets, Ras and HMG2A. Knockdown of either protein separately revealed that Ras was responsible for enhancing cell proliferation, while HMGA2 suppressed differentiation of BCSC. In this context let-7 is regulated by an inflammatory pathway that is involved in epigenetic switching of cells into a cancerous phenotype (263). This pathway is triggered by Src, which, via activation of NF$\kappa$B, induces the expression of Lin-28, an inhibitor of pri-let-7 processing (448). Subsequently, as lower levels of let-7 enhance the expression of IL-6, which also activates NF$\kappa$B, the pathway becomes perpetually activated. Other miRNAs that were discovered by differential profiling of BCSC included the followiing: miR-200c-miR-141, miR-200b-miR-200a-miR-429, and miR-183-miR-92-miR-1182, which were downregulated in both BCSC and embryonic stem cell carcinoma (559). This group of miRNAs was also lower in normal breast stem cells relative to the more differentiated progenitor cells. miR-200c/miR-200b/miR429, which all have the same seed sequence, target and suppress Bim1, a protein known for its function in stem cell renewal (470). Thus, as predicted, overexpression of miR-200c inhibited colony formation by BCSC, through suppression of Bmi1, and completely inhibited their tumorigenic capacity in vivo (559).

### 2. Tumor growth (Figure 9)

Early studies showing the differential expression pattern of miRNAs in breast cancer tissue revealed that some of the more significantly upregulated included miR-21 and miR-155, and those downregulated included miR-125b and miR-145 (267). Consistent with a tumor-promoting function, miR-21 was found to target and inhibit TPM1 (716), PDCD4 (184, 391), and maspin (717). While PDCD and TPM1 are generally known for their tumor suppressive properties, maspin has been implicated in the inhibition of tumor invasion by inducing upregulation of $\alpha$3 and $\alpha$5 integrins in breast cancer cells (540). In agreement, ectopic

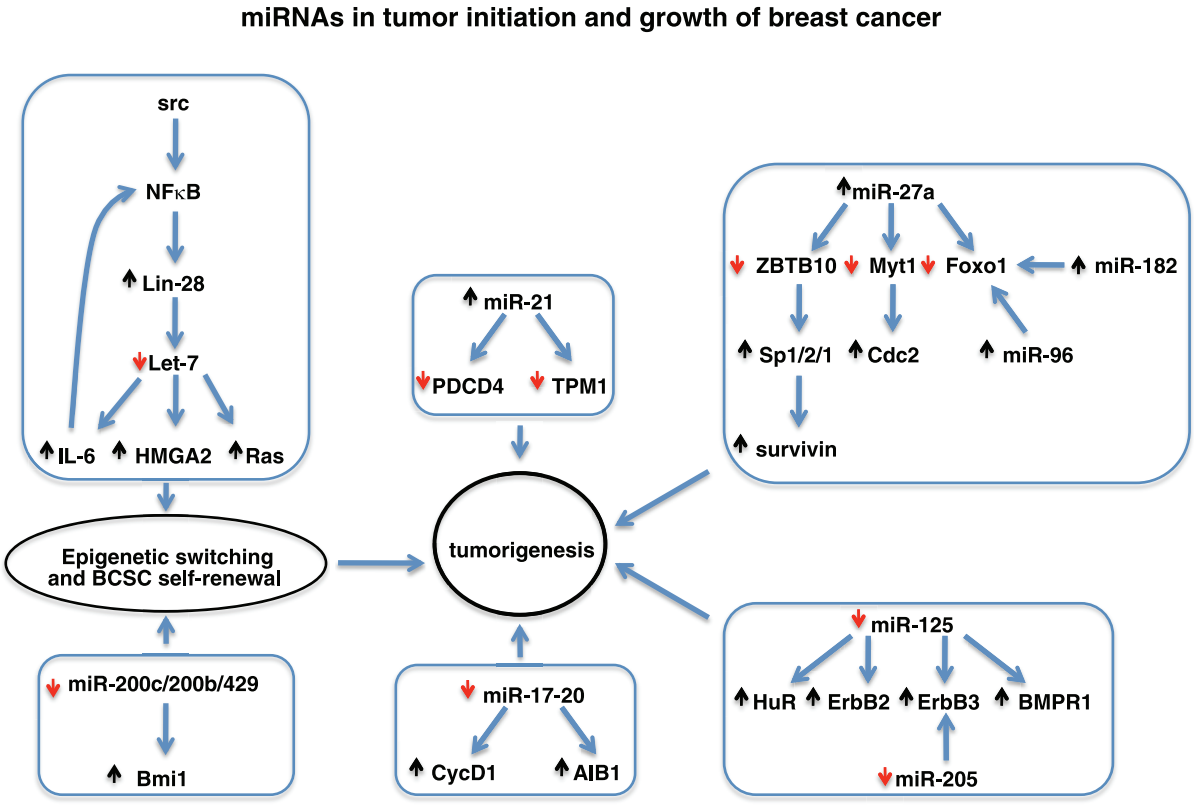


**FIGURE 9** A diagram showing miRNAs and their targets in initiation and growth of breast cancer. The diagram displays the different miRNAs and their targets that are involved in breast cancer stem cells (BCSC) self-renewal and tumor growth. This also includes some of the identified upstream molecules that regulate their expression. Upregulation or downregulation of a specific miRNA is represented by an upward (black) or a downward (red) arrow, respectively. The changes in the expression levels of a target gene inversely correlate with that of the targeting miRNA and are similarly represented by an up or down arrow. All listed targets have been validated. The targets listed include the following: v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (src), nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (NFκB), interleukin-6 (IL-6), high mobility group AT-hook 2 (HMGA2), Ras oncogene, signal transducer and activator of transcription 3 (STAT3), programmed cell death 4 (PDCD4), tropomyosin 1 (TPM1), zinc finger and BTB domain containing 10 (ZBTB10), myelin transcription factor 1 (Myt1), forkhead box O1 (Foxo1), Sp1 transcription factor, cell division cycle 2 protein (Cdc2), Hu antigen R (HuR), v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 (ErbB2) and homolog 3 (ErbB3), bone morphogenetic protein receptor type 1B (BMPR1B), cyclin D1 (CycD1), and amplified in breast cancer 1 protein (AIB1).

expression of miR-21 enhanced breast cancer cell growth both in vitro and in vivo (184, 391, 560) and HER2-mediated cell invasion (251), and promoted metastasis (717). Conversely, its knockdown in an invasive breast cancer line inhibited metastasis, although it had no effect on tumor growth (717). In addition to its pro-growth and -metastasis effects, miR-21 also indirectly induces upregulation of Bcl-2 and, thus, exerts an antiapoptotic function (560). In concordance, miR-21 is one of the few miRNAs that correlates with advanced clinical stage, lymph node metastasis, and poor survival in breast cancer patients (685). In addition, miR-27a is another oncomir that is highly expressed in breast cancer cells (226, 421) and targets the zinc finger protein ZBTB10 (421) and the proapoptotic protein Foxo1 (226). Via suppressing ZBTB10, it induces upregulation of the transcriptional enhancers Sp1,3,4, which, in turn, increase expression of the antiapoptotic protein survivin (421). Moreover, by targeting the cell cycle inhibitor Myt-1 (421), miR-27a may promote tumor growth. These effects may be enhanced by the upregulation of miR-96 and miR-182, which also target Foxo1 (226).

In contrast to the upregulation of miR-21 and miR-27 and their oncogenic functions, the miR-17~20 cluster (247, 698) and miR-125a (224) are reduced in breast cancer. miR-17~20 cluster inversely correlates with the expression of its target cyclin D1 (698) and amplified in breast cancer 1 (AIB1) (247) and, thereby, inhibits estrogen-dependent and -independent cell proliferation of breast cancer cells (247, 698). Meanwhile, miR-125a inhibits cell proliferation and migration via targeting the RNA-binding protein HuR (224), and ErbB2 and ErbB3 family members of the EGFR (539). Moreover, the discovery of a germ line mutation in miR-125a (362) and a SNP in the miR-125b-targeted site of the 3′-UTR of bone morphogenic receptor type 1B

(BMPR1B) (522) that are associated with breast cancer, confirms its role in the underlying pathogenesis.

### 3. Metastasis (Figure 10)

EMT of cancer cells is one of the mechanisms that increases the cell's motility and invasiveness and is characterized by loss of proteins involved in cell-to-cell attachments (21). E-cadherin is one of these proteins that is suppressed by the transcriptional regulators snail, zinc-finger E-box-binding homeobox 1 (ZEB1/TCF8/δEF1), and ZEB2/SIP1; however, the mechanism of their regulation is not fully understood yet (reviewed in Ref. 480). miRNAs provide one mode of regulation for these critical factors as seen in many cancers. Downregulation of miR-200c is implicated in EMT and cell invasion by releasing ZEB1 from its inhibitory effect and,

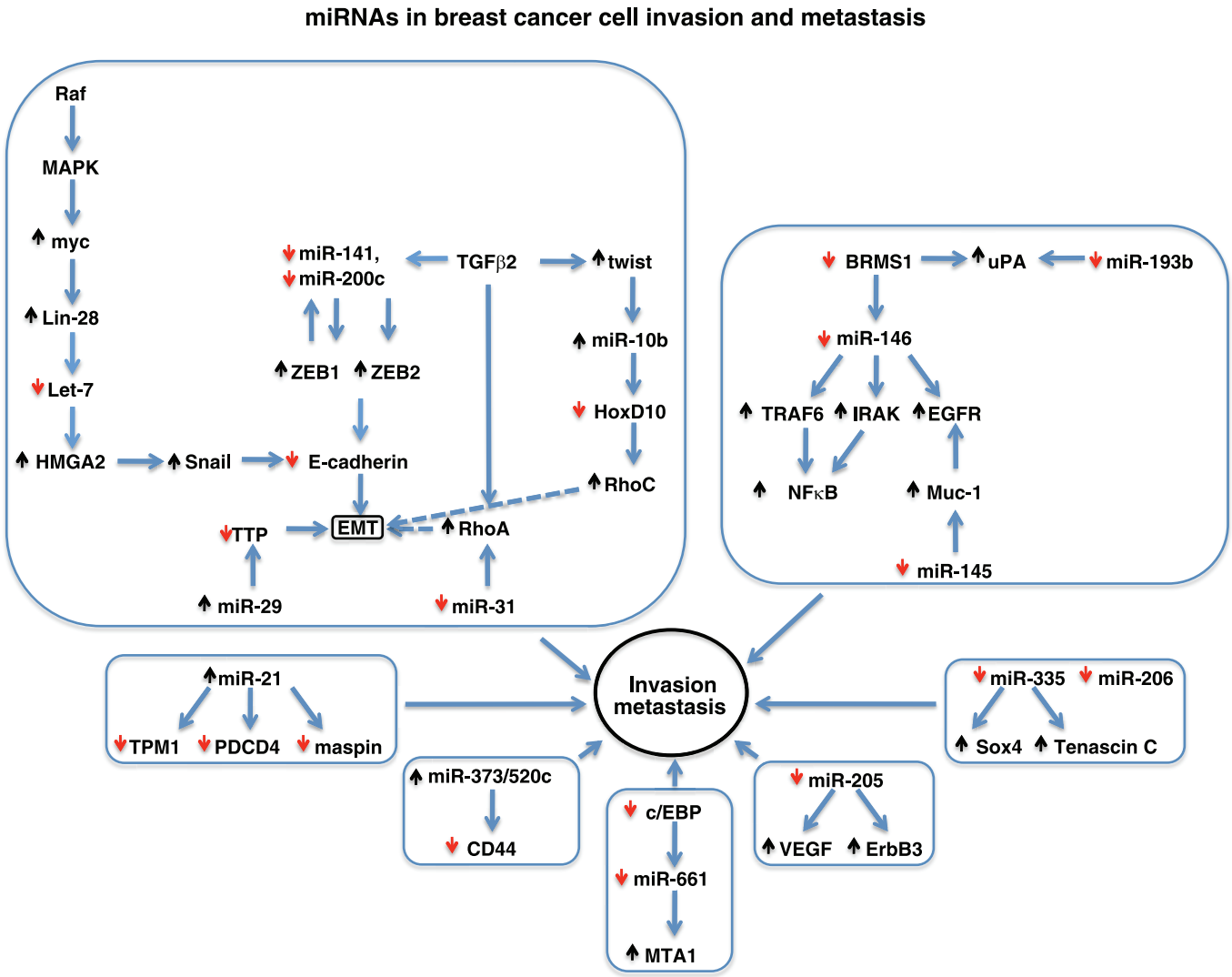


**FIGURE 10** A diagram showing miRNAs and their targets in breast cancer metastasis. The diagram displays the different miRNAs and their targets that are involved in breast cancer cell invasion and metastasis. This also includes some of the identified upstream molecules that regulate their expression and the convergence of multiple miRNAs on endothelial-to-mesenchymal transition (EMT). Upregulation or downregulation of a specific miRNA is represented by an upward (black) or a downward (red) arrow, respectively. The changes in the expression levels of a target gene inversely correlate with that of the targeting miRNA and are similarly represented by an up or down arrow. All listed targets have been validated. The targets listed include the following: Raf oncogene, mitogen-activated protein kinase (MAPK), myelocytomatosis oncogene (myc), high mobility group AT-hook 2 (HMGA2), E-cadherin, ZEB1/2, TTP, TGFb1, ras homolog gene family, member A/B (RhoA/C), homeo box D10 (HoxD10), breast cancer metastasis-suppressor 1 (BRMS1), plasminogen activator, urokinase (uPA), TNF-receptor-associated factor 6 (TRAF6), interleukin-1 receptor-associated kinase 1 (IRAK), epidermal growth factor receptor (EGFR), nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (NFkB), mucin 1(Muc-1), tropomyosin 1 (TPM1), programmed cell death 4 (PDCD4), maspin, CD44 antigen, metastasis associated 1 (MTA1), SRY-box containing gene 4 (Sox4), Tenascin C, vascular endothelial growth factor (VGEF), and v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (ErbB3).

thus, suppressing the expression of E-cadherin in breast cancer cells (256). Indeed, overexpression of miR-200c induces differentiation of breast cancer cells into epithelial cells and reduces cell migration and invasion equivalent to the effect seen on knockdown of ZEB1 (63). Meanwhile, miR-141, another miR-200 family member, targets TGF-$\beta$2, which is an inducer of EMT. Conversely, ZEB1 directly suppresses the promoter of miR-141/-200b/-200c (63), creating a double negative feedback loop. Upon closer examination, it was discovered that miR-200a/141 and miR-200b/200c/429 (each group having common seed sequences) target both ZEB1 and ZEB2 through multiple target sites for each within their 3′-UTR (215). As expected, miR-200 family members are specifically lost in metastatic breast cancer cells that lack E-cadherin. Thus the miR-200 family is involved in both cancer initiation and invasion.

To further identify genes that are involved in breast cancer metastasis, a differential profiling study was performed on a human breast cancer cell line and its highly metastatic derivatives (602). This uncovered two miRNAs, miR-335 and miR-206, whose expression is selectively reduced during metastasis and that positively correlate with a low metastasis free survival in patients. Indeed, replenishing these miRNAs reduced metastasis in vivo but did not inhibit tumor growth or cell proliferation, which is in contrast to miR-126. miR-126, which was discovered in the same screen, also inhibits metastasis; however, it is unclear if its effect is direct or secondary to inhibition of the tumor's growth. It was also determined that the function of miR-335 and miR-206 are not due to inhibition of EMT, but rather a consequence of an independent decrease in cell migration. miR-335 targets included the transcription factor Sox4 and the extracelluar matrix protein tenascin, both of which are required for cell migration.

Another miRNA that was identified in metastasis, based on its consistent downregulation that was reported by separate differential expression studies, is miR-31. While exhibiting ~4-fold downregulation in nonmetastatic cancer cells relative to noncancerous cells, it is >100-fold reduced in metastatic cells (624). It is also lower in metastatic cells relative to their tumor of origin, and its expression inversely correlates with metastasis in human samples. Although overexpression of miR-31 in implanted tumors resulted in ~1.5-fold larger tumors, they were well encapsulated with reduced metastatic capacity. In addition to that, miR-31-expressing cancer cells had reduced capacity for colonization and proliferation in metastatic sites. A reverse effect was elicited by knockdown of miR-31 in noninvasive cells. In this context, miR-31 was shown to target and inhibit a consortium of genes that included frizzled (FZD3), integrin $\alpha$5 (ITGA5), matrix metalloproteinase 16 (MMP16), radixin (RDX), and RhoA. In particular, replenishing RhoA alone was sufficient for completely reversing miR-31-induced inhibition of invasion or metastasis both in vitro and in vivo. RhoA is also a downstream effector of TGF-$\beta$-induced EMT (45) and, thus, may be involved in mediating its effect on the miR-200 family.

Let-7 has been shown to function as a tumor suppressor, at least partly through suppression of Ras (282). In breast cancer, let-7 suppresses proliferation, tumorgenicity, and metastasic potential of breast cancer stem cell (695). In addition to the inflammatory signaling pathway described above, let-7 is also downregulated by the Raf-MAPK-Myc-Lin-28 pathway that is suppressed by Ras kinase inhibitory protein (130). By targeting HMGA2, a chromatin remodeling protein, it suppresses the expression of Snail, which is expressed in metastatic tumors and has been implicated in the mechanism for EMT by directly suppressing E-cadherin (73). This further explains how downregulation of let-7 may suppress breast cancer cell metastasis (695).

Breast cancer metastasis suppressor 1 (BRMS1) is a known suppressor of breast cancer metastasis (548), functioning via inhibiting the activities of various genes including NF$\kappa$B (110), EGFR (255), and urokinase-type plasminogen activator (uPA) (109). Recently, BRMS1 was shown to induce miR-146, which directly suppresses expression of EGFR and has the capacity to independently inhibit metastasis, suggesting that it may mediate BRMS1's inhibitory function (254). In consensus, miR-146 also inhibits NF$\kappa$B activity by suppressing its regulators TNF receptor-associated protein 6 (TRAF6) and interleukin-1 receptor associated kinase (IRAK1) (44). In parallel to its regulation by miR-146, EGFR is indirectly induced as a result of downregulation of the tumor suppressor miR-145 and the increase in its target mucin 1 (520), which prevents its degradation (490). Mucin 1 proved sufficient for enhancing cell invasion, and its effect is overcome by overexpression of miR-145 (520), although whether EGFR mediates its effect has not been determined. miR-193b is also implicated in this network as it is reduced in breast cancer cells, thus releasing uPA from its inhibition (364). Accordingly, overexpression of miR-193b inhibits breast cancer cell growth and metastasis, but there is no evidence yet if it is also induced by BRMS1.

In contrast to miRNAs that inhibit metastasis, others were found to promote it. miR-10b is specifically upregulated in metastatic breast cancer cells (397). It is directly induced by Twist, which itself plays an essential role in EMT-induced metastasis (688). Both gain- and loss-of-function experiments proved that miR-10b is both necessary and sufficient for specifically inducing cell invasion and metastasis in vitro and in vivo (397). HoxD10 was validated as a direct target of miR-10b. This protein belongs to a family of homeobox genes that are involved in maintenance of the differentiated cell status and are, accordingly, generally downregulated in invasive breast cancer tissue (402). In particular, HoxD10 was shown to inhibit breast

cancer cell invasion (78). Suppression of this gene's expression and derepression of its transcriptional target RhoC are necessary events in mediating miR-10's metastatic effect (397). This pathway potentially converges on EMT via RhoC, which induces EMT in colon cancer cells (28).

In addition to miR10b, upregulation of miR-373 and miR-520c also specifically promote metastasis (250). These miRNAs were discovered through a gain-of-function screen that involved 450 miRNAs. Introducing miR-373 or miR-520c into MCF-7 resulted in enhancing cell migration and invasion and in vivo metastasis, but not proliferation. Moreover, miR-373/520c were higher in metastastic lymph node cells than in primary breast cancer tumors from which they originated. A main target that is suppressed by both these miRNAs, as they both have the same seed sequence, is the metastasis-related hyaluronan receptor CD44. However, studies on this molecule have overwhelmingly demonstrated a prometastatic role of CD44 in various cancer cells and has been accordingly a target of therapeutic interventions [reviewed by Toole et al. (616)]. Thus the fact that miR-373/520c promotes metastasis by suppressing CD44 does not reconcile well with the established function of this protein. It should be noted, though, that there are a few studies that demonstrated an anti-metastatic role for CD44, including Huang et al. (250), who show that knockdown of CD44 in breast cancer cells enahnces invasiveness, whereas overexpressing it reduces miR-373-induced invasion. However, a role for other miR-373/520c targets cannot be excluded.

Several other miRNAs are also involved in breast cancer metastasis through regulating crucial molecules that are involved in the process. For instance, miR-205 is downregulated in human breast cancer resulting in increased expression of its target ErbB3, thus activating the AKT pathway (266, 669). Consequently, reintroducing miR-205 in cancer cells reversed these effects and reduced colony formation. Moreover, it suppressed lung metastasis in vivo (669). Although another study has shown that miR-205 targets the EMT genes ZEB1 and ZEB2, its expression levels in invasive ductal versus metaplastic breast cancer tissues was variable and did not correlate with E-cadherin levels (215). miR-661 also participates in metastasis by declining during breast cancer progression as a consequence of the downregulation of its transcriptional activator c/EBP$\alpha$ (504). It targets metastatic tumor antigen 1 (MTA1), which is a transcriptional corepressor that induces deacetylation of histones, p53, and Hif-1$\alpha$, and promotes a more aggressive phenotype of cancer cells (615). Thus, as predicted, overexpression of miR-661 in breast cancer cells inhibits migration, invasion, and tumor size in vivo (504). miR-29, on the other hand, is upregulated in the invasive mesenchymal breast cancer cells but not in epithelial cell lines (203). It enhances EMT-dependent metastasis and directly suppresses tristetraprolin (TTP). TTP is an RNA-binding protein that destabilizes AU-rich mRNAs, which has been implicated in tumor suppression by targeting genes such as IL-3 (580), Hif-1$\alpha$ (301), and Cox2 (56). Knockdown of this protein was shown to enhance EMT; however, direct proof that it indeed mediates miR-29a effects has not been presented (203).

#### *4. Estrogen receptors (ER) in breast cancer*

Most breast tumors express ER$\alpha$ and ER$\beta$ receptors, which have opposing effects on mammary development and differentiation; ER$\alpha$ enhances proliferation while ER$\beta$ inhibits it (355). Several studies have identified miRNAs that are regulated by estrogen in cancer cells. These included miR-17~92 and its paralog miR-106a-363 that are upregulated by E2 in MCF-7 cells and function as negative regulators of the ER receptor and its coactivator AIB1 (80), miR-21, and let-7 (42). However, upregulation of miR-17~92 and let-7 contradict other findings that show that they are downregulated in breast cancer (247, 695, 698). Controversially, Maillot et al. (400) reported that estrogen induces only downregulation of miRNAs in MCF-7 cells (400). In particular, miR-26a and miR-181a strongly inhibit estrogen-induced proliferation and directly target and suppress the progesterone receptor. On the other hand, miRNAs that regulate the ER$\alpha$ include miR-206 (3) and miR-221/222 (3), which is consistent with the finding that miR-221/222 is upregulated in tamoxifen-resistant cancer cells (427).

## XIV. MicroRNAs IN OVARIAN CANCER

In general, ovarian cancer (OC) is associated with a larger fraction of downregulated (88.6%) than upregulated miRNAs (706). Indeed, it was found that ~37% of miRNA genes are encompassed in genomic regions that are subject to DNA copy abnormalities in OC (705), while ~36% exhibit epigenetic silencing (706). However, studies have differed on the role of Dicer and Drosha in this preferential downregulation of miRNAs. The consensus, though, is that low Dicer expression in epithelial and serous ovarian cancer is associated with advanced tumor stage and low survival (169, 420). Some specific prognostic markers that have also been reported include low levels of the miR-200 family that predicted poor survival (248) and low level of let-7i that was associated with shorter progression-free survival of end-stage patients (689). Moreover, HMGA2 was identified as a let-7 target that was elevated in OC, and together their reciprocal pattern of expression was associated with a <5-yr progression-free survival (552). It should be noted though that the expression of let-7 and HMGA2 did not differ in primary versus metastatic tumors (473). Consistent results were observed with lin-28b, a negative regulator of let-7 processing, where its expression positively correlated with low progression-free survival (387). In addition, let-7 is also silenced by DNA methylation (386).

Specific miRNAs that are downregulated in OC have been identified as inhibitors of cell growth; those include miR-34c, miR-15/16, miR-9, and miR-199a. P53-deficient OC cells are low in miR-34b and miR-34c, which are transcriptional targets of p53 (118). Moreover, low levels of miR34c correlate with decreased survival in serous OC (338). Replenishment of these miRNAs reduces cell proliferation; however, predicted targets remain to be validated (118). Reduced miR-15 and miR-16's expression levels inversely correlate with their target Bmi-1 and, thus, inhibit proliferation when they are ectopically expressed (43). A similar function is observed via downregulation of miR-9 and derepression of its target NF$\kappa$B1 (223), whose activity may be further enhanced by downregulation of miR-199a and upregulation of its target IKK$\beta$ (92). On the other hand, downregulation of the miR-200 family members directly correlates with the expression of E-cadherin in primary serous papillary OC, as it has been shown to directly inhibit the expression of E-cadherin's repressors ZEB1 and ZEB2 (472). The latter cluster of miRNAs, in addition to an increase in AT-rich interactive domain 3B protein (ARID3B) as a result of reduced miR-125 levels (124), are likely to contribute to EMT and enhance OC cell migration, although this has not been confirmed yet.

## XV. MicroRNAs IN PROSTATE CANCER

miRNAs profiling of prostate tumors uncovered the dysregulation of some of the usual culprits, including downregulation of let-7, miR-145, miR-141, miR-125, miR-1, miR-133, miR-106b, and miR-16 (12, 466, 491). The miR-15/16 cluster, which is present in the 13q14 chromosomal region, is increasingly lost during the progression of cancer of the prostate (CaP). Reduced levels of miR-15/16 are associated with an increase in the protein levels of their targets Bcl2, cyclin D1, and WNT3A (53) that is known to enhance CaP growth and stem cell self-renewal (46, 363, 635). Indeed, knockdown of this cluster in a mouse model results in prostate hyperplasia that is associated with an increase in cyclin D1 and Wnt3A (53). Likewise, its knockdown in normal prostate cells increases survival, proliferation, migration, and tumorigenic capacity and invasion in vivo. Conversely, overexpression of miR-15/16 suppresses tumor growth and induces regression. Another miRNA that is frequently lost in CaP is miR-101; it is absent in 37% of local CaP tumors and 66% of metastatic cells (629). This inversely correlates with upregulation of its target, enhancer of zezte homolog 2 (Ezh2), a methyltransferase subunit of the polycomb repressive complex 2. This protein is highly expressed in hormone-refractory, metastatic, prostate cancer and promotes cell proliferation (630). Accordingly, suppression of Ezh2 expression by miR-101 inhibits cell proliferation and invasion and reduces global genomic trimethyl H3k27 levels (629). Additional tumor suppressors that are downregulated in CaP include miR-331–3p and miR-449a, which contribute to tumor growth by releasing their targets ErbB2 (164) and HDAC1 (453), respectively, from their inhibitory effect.

Another array of miRNAs that have proapoptotic functions are also dysregulated in CaP and enhance cell survival. These include downregulation of miR-34, which is especially reduced in p53-deficient CaP cells, as it is induced by p53 and mediates its apoptotic effects (188, 511); downregulation of miR-330, which suppresses E2F-1 and E2F-1-medited AKT phosphorylation (339); upregulation of miR-20a, which suppresses E2F2 and E2F3 and prevents their excessive accumulation and, thus, apoptosis (590); upregulation of miR-125b, which inhibits the expression of the proapoptotic protein Bak1 (557), in addition, to the upregulation of miR-21, which is an established suppressor of apoptosis (361). In CaP cells, miR-21 is induced by stimulation of androgen receptors and mediates hormone-dependent and -independent cell growth (508). In contrast, though, miR-21 enhances cell migration and invasion but not proliferation in androgen-independent cell lines, where it targets myristoylated alanine-rich C kinase substrate (MARKCS) (361). However, the set of targets that distinguish miR-21's functions in androgen-dependent vs. -independent cells has not been determined. In contrast to miR-21, miR-221 increases in androgen-independent tumors (586) and positively regulates cell proliferation, colony formation, and tumor growth through directly suppressing $p27^{kip}$ (193, 419). In contrast, it is downregulated in 98% of metastatic CaP tumors and is associated with poor prognosis (576). However, the set of targets that distinguish these two effects of miR-221 have not been determined. Other miRNAs involved in cell invasion of CaP include miR-146a, which is reduced in androgen-independent CaP cells (369). It inhibits proliferation and hyaluronan-induced cell invasion through preventing upregulation of Rho-activated protein kinase 1 (ROCK1). On the other hand, PDGF induces EMT and cell invasion through downregulation of miR-200 family member, which targets ZEB1 and ZEB2 (310), while downregulation of miR-205 participates in this process by derepressing a panel of targets that include ErbB3, E2F1, E2F5, ZEB2, and PKC-$\varepsilon$ (194).

## XVI. EXPLOITING MicroRNAs IN THERAPEUTICS

miRNAs are characterized by targeting multiple, functionally related, versus single, genes, which renders them a potentially powerful therapeutic tool. As an example, it proved more functionally effective to simultaneously, rather than individually, inhibit the cell cycle-related miR-16 targets (373). One of the tools that has been developed for reducing a miRNA's levels in vivo is a specific antisense oligonucleotide. Indeed, various forms of modified antisense miRNAs (antimiRs), with (antagomirs) or without a cholesterol conjugate, have been applied in vivo with great success. Hutvagner et al. (258) first reported that 2′-*O*-methyl-modified oligonucleotides successfully inhibited let-7 function in *Drosophila*, whereas Esau et al. (165) observed inhibition of liver miR-122 in mice by using 2′-*O*-methoxyethyl phosphorothioate-modified oligodeoxy-

nucleotides. Likewise, injection of 2′-*O*-methyl, phosphorothioate, 5′-cholesterol-conjugated antisense miR-122 modified oligonucleotides "antagomirs," effectively inhibited miR-122 in the liver (320). These antisense oligos appear to function by binding to the corresponding mature miRNA within the cytoplasmic compartment and induces its degradation (319). There are several examples that have been mentioned throughout this review that demonstrate the successful use of antisense miRNA constructs in treating animal disease models. This includes alleviation of cardiac fibrosis and hypertrophy by intravenous injection of a miR-21 antagomir, as a result of derepressing the expression of its target SPRY1 and increasing myofibrolast apoptosis (612). Alternatively, Elmen et al. (161) used intravenous injection of a 16-nt-long antisense, unconjugated, locked-nucleic-acid-modified oligonucleotide (LNA-antimiR), to deplete miR-122 in primates' liver. This strategy proved effective in reducing serum (2.6 logs) and liver (2.3 logs) HCV, with no evidence of viral resistance or side effects (331).

On the other hand, miRNAs are also frequently downregulated in disease, in which case a therapeutic approach would require replacement of specific miRNAs. According to our knowledge of a miRNA's processing and function, this would require the double-stranded form of the molecule for proper assembly of the "driver" strand into the miRISC. This kind of construct was dubbed miRNA mimic or mimetic. The double-stranded design, versus the single-stranded mature miRNA, did indeed prove necessary for efficient incorporation of the mature miRNA into the miRISC (182, 221). As proof of concept, Wiggins et al. (662) showed that intravenous or intratumoral injection of a miR-34a mimic, in a lipid-based vehicle, in a mouse model of non-small-cell lung cancer, induced apoptosis and significantly inhibited tumor growth. Similarly, direct injection of Let-7 mimics into a lung cancer xenograft reduced tumor burden by 66% (619). As an alternative, the primary or precursor sequence of miRNAs under the control of a constitutive or tissue-specific promoter can be delivered in vivo using viral vectors. This has been successfully demonstrated by intranasal delivery of a Let-7-expressing lentivirus in mice, which resulted in a 75% decrease in size of K-ras-induced tumors (619). On the other hand, Kato et al. delivered miR-26a intravenously via an adenoassociated virus (315). One of the criteria they used was that the miRNA they chose would be normally highly expressed in all organs and reduced only in the tumor, as they predicted it would be generally well tolerated. Promisingly, they found that miR-26a, which targets cyclins D2 and E2, reduced tumor burden in the liver, as a result of reduced proliferation and increased apoptosis of the tumor cell. Conversely, one can envision the delivery of viral vectors that express specific antisense miRNA-targeting constructs, such as the "eraser" (497, 534) or the "sponge" (152) for reducing the levels of upregulated miRNA in diseased organs, which awaits testing.

## XVII. CONCLUSION

In conclusion, while much has been done to elucidate the functions of individual miRNAs during health and disease, much more remains to be discovered. Specifically, the full spectrum of genes that a single miRNA targets in a given context, how these targets functionally interact, and the extent to which each contributes to the miRNA's observed functionality. In addition, since a single mRNA could be simultaneously regulated by multiple miRNAs, the collaborative effects of these miRNAs in a given function need to also be explored. These details will further explain the functional significance of miRNAs and aid in planning for future therapeutic targeting of a miRNA.

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## DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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