Review

The Role of miRNAs in Regulating Gene Expression Networks

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Abstract

MicroRNAs (miRNAs) are key regulators of gene expression. They are conserved across species, expressed across cell types, and active against a large proportion of the transcriptome. The sequence-complementary mechanism of miRNA activity exploits combinatorial diversity, a property conducive to network-wide regulation of gene expression, and functional evidence supporting this hypothesized systems-level role has steadily begun to accumulate. The emerging models are exciting and will yield deep insight into the regulatory architecture of biology. However, because of the technical challenges facing the network-based study of miRNAs, many gaps remain. Here, we review mammalian miRNAs by describing recent advances in understanding their molecular activity and network-wide function.

Introduction

MicroRNAs (miRNAs) are ~22-nucleotide RNAs that post-transcriptionally repress gene expression by base pairing to mRNAs. More than half of all mRNAs are estimated to be targets of miRNAs, and each miRNA is predicted to regulate up to hundreds of targets. Consistent with this pervasive activity, miRNAs regulate a broad range of processes, including proliferation, differentiation, and apoptosis. These short RNAs are particularly critical during development, their total loss in the embryo leading to lethality. Although many studies focus on binary miRNA–target interactions in defining phenotypes, it is becoming increasingly evident that each miRNA exerts its influence by targeting multiple functionally related genes that constitute gene expression networks. In this review, we provide a network-level perspective of mammalian miRNAs and describe their genomic organization, molecular activity, and biological function.

The Molecular Biology of miRNAs

Biogenesis and genomic organization

The biogenesis of miRNAs has been reviewed extensively elsewhere[1] and is summarized briefly here. Genes encoding miRNAs are transcribed by RNA polymerase II into long primary transcripts (pri-miRNAs) that are processed by the RNase III enzyme Drosha to yield precursor miRNAs (pre-miRNAs)[2] (Fig. 1). Pre-miRNAs are subsequently transported into the nucleus by Exportin 5[3–5] and then processed by another RNase III enzyme, Dicer, to yield a mature miRNA as well as a star strand that is degraded.[6–10] The miRNA is then loaded into an Argonaute protein within the RNA-induced silencing complex (RISC), the effector complex that mediates repression of targets. Within the RISC, the miRNA acts as a guide strand conferring target specificity through a sequence termed the "seed", which spans positions 2–7 of the miRNA.[11–14] miRNAs with identical seed sequences are grouped into families and, since target specificity is typically dictated by the seed, members of a family generally repress the same mRNAs. Examples of targeting mediated by regions outside the seed have also been reported but are uncommon.[15,16]

Genes encoding miRNAs belong to several classes of gene structures, the result of various events during the course of evolution (reviewed in detail elsewhere[17]). miRNAs can be transcribed from intergenic regions, where an individual miRNA gene or a cluster of miRNAs forms an independent

transcriptional unit, or from introns of coding genes. Based on the exhaustive annotation described in a landmark study by Chiang *et al.*, 38% of murine miRNAs fall within introns of mRNAs.[18] In most cases, the miRNA is processed from the intron of the host transcript; thus, the miRNA and host gene are coordinately expressed. Additionally, multiple miRNA hairpins are often encoded as clusters within a single primary transcript. In mammals, 61% of miRNAs are expressed from polycistronic clusters.[18] These clusters can encode multiple miRNA seed families. While expression between clustered miRNAs is strongly correlated, it is not absolute, indicating regulation at the level of processing.[18]

The clustered organization of miRNAs suggests shared biological function among unrelated miRNAs present in the same primary transcript. For example, let-7[11] and mir-125/lin-4,[12,19] both of which control the timing of development in worm, are clustered in most animals (though not in *Caenorhabditis elegans*), in line with a common and conserved function in developmental timing.[20] Similarly, the miR-1-2~133 cluster encodes two miRNAs that are not related by seed but are related by function, each regulating muscle development and myogenic gene expression networks.[21] Additional evidence for shared function between clustered miRNAs is provided in the section below describing miR-17~92 and c-Myc.

Although clustering may serve an important biological function, it nevertheless poses an obstacle when interpreting classical genetic loss-of-function studies in which deletion of a miRNA gene ablates expression of multiple clustered miRNA seed families. In such instances, the phenotype may not be attributable to any single seed family and, therefore, would require

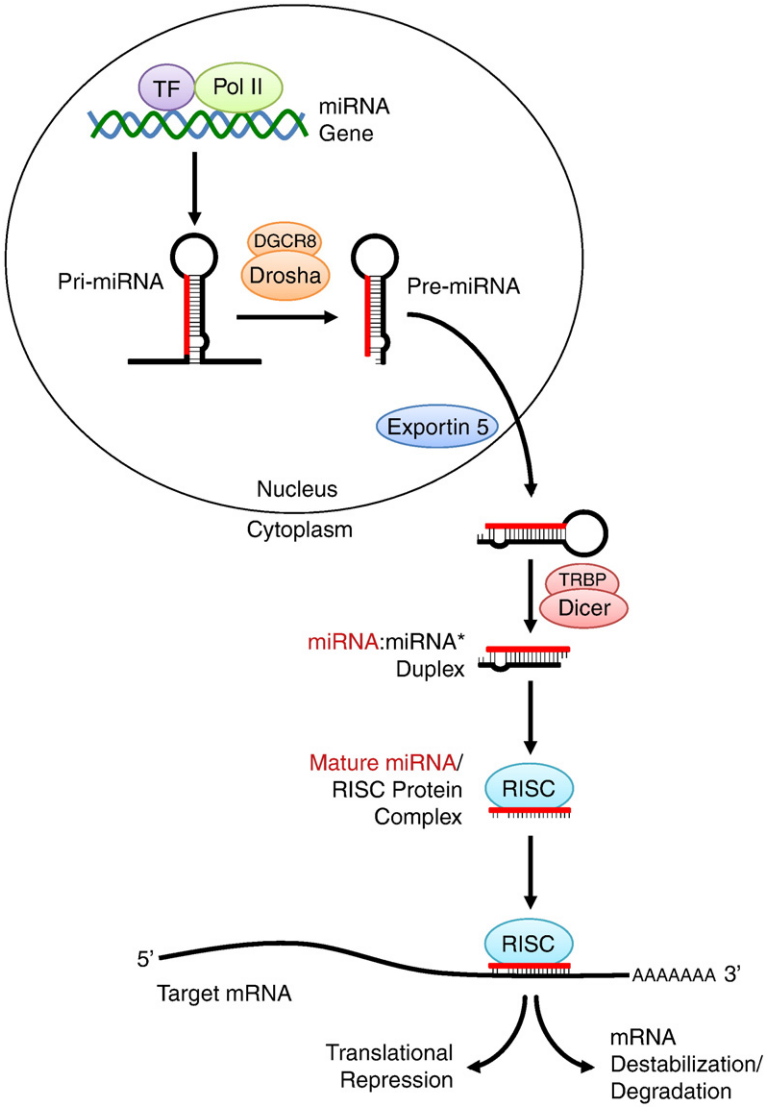

Fig. 1. The miRNA biogenesis pathway. MiRNA genes are transcribed by RNA polymerase II, in combination with specific transcription factors (TF), as long primary transcripts (pri-miRNA). These transcripts are then processed in the nucleus by the RNase III enzyme Drosha, in complex with DGCR8, into pre-miRNAs, which are exported into the cytoplasm by Exportin 5. Pre-miRNAs are processed by the RNase III enzyme Dicer, in complex with TRBP, into a duplex consisting of a guide strand (miRNA) and passenger star strand (miRNA*). The mature miRNA is loaded into the RISC and acts as a guide strand that recognizes target mRNAs based on sequence complementarity. The RISC subsequently represses targets by inhibiting translation or promoting destabilization of target mRNAs.

phenotypic rescue with the expression of individual, transgenic miRNAs. Examples of such rescue experiments are provided below in the characterization of miR-17~92 in mouse tumor models. Although technically challenging, selective genetic ablation of an individual miRNA within a cluster can be achieved, as reported for the deletion of miR-1-2, which is clustered with miR-133.[22]

A single miRNA seed family can also be expressed from multiple genomic loci, exemplified by the expression of let-7 from eight different loci in the mouse and human genomes. This organization may provide greater flexibility in modulating miRNA activity, for example, by allowing promoter- and context-specific expression of different members of a single seed family. Like clustering, this genomic dispersion complicates genetic analysis of certain miRNA families. Several studies have reported the deletion of multiple loci encoding paralogous clusters of miRNAs, for example, for miR-17~92 or miR-34 and their related clusters as described below. Coupled with the fact that such loci can also include clusters of unrelated miRNA seed families, genetic analysis of an isolated miRNA seed family can, in some cases, be unfeasible. The use of "sponge" constructs that titrate miRNA activity based on seed complementarity may circumvent these technical challenges.[23–25] In total, the functional impact of the genomic organization of miRNAs is underexplored but made feasible by the groundwork of tools and knowledge established by existing studies.

Activity of the RISC

The RISC is the key effector complex of the RNAi pathway. When loaded with a guide strand, it can inhibit mRNAs by two different mechanisms. When the guide strand and target are perfectly complementary, as observed for small interfering RNA-mediated RNA interference, the slicer activity of Argonaute 2 (Ago2) cleaves the target between nucleotides complementary to positions 10 and 11 of the guide strand, leading to rapid decay of the resulting products.[26] When guide and target are imperfectly complementary, as is the case for almost all miRNA–target interactions in animals, the RISC initiates a cascade of inhibitory events that direct targets to canonical degradation pathways.[27] First, the mRNA is translationally inhibited through a poorly understood mechanism that likely occurs at the step of translational initiation.[28] Then, the GW182 proteins (TNRC6A-C), which are direct binding partners of Argonaute and well-established members of the RISC, recruit two deadenylase complexes, namely, PAN2–PAN3 and CCR4–NOT, to deadenylate the targeted mRNA.[29–31] Finally, the deadenylated transcript is decapped by DCP2 and degraded by XRN1, the cytoplasmic 5′-to-3′ exonuclease.[32]

The seed-based target complementarity by which mRNAs are bound and inhibited allows combinatorial diversity in gene regulation. This property is used by several algorithms, such as TargetScan,[14] miRanda,[13] and PicTar,[33] to predict targets. A single miRNA seed family may be predicted to target 100–1000 mRNAs. Computational analysis has revealed enrichment for functional pathways among targets of individual seed families.[34] Experimentally, the pleiotropy of miRNA activity has been demonstrated by transfection or overexpression of miRNAs followed by gene expression profiling.[35]

In addition to the regulation of multiple mRNAs by a single miRNA seed family, a single target may itself possess multiple seed matches for a given miRNA family, thereby leading to enhanced repression. Notable examples include Hmga2[36] and Igf2bp1,[37,38] which are strongly repressed by multiple let-7 target sites in their 3′ untranslated regions (UTRs). Additionally, a single mRNA can be repressed by multiple miRNA families. Computational analysis has revealed that co-targeting of mRNAs by functionally related miRNAs is prevalent, particularly for clustered miRNAs.[34] While some experimental evidence supports this observation for certain miRNA families,[39] a systematic experimental investigation of co-targeting relationships has not been carried out.

Structure of Argonaute

Argonaute is the catalytic engine of RISC and serves as a platform to recruit additional regulators of mRNA stability. Therefore, intense effort has been devoted to understanding its function at the structural level. Until recently, structural insights were obtained either from crystals of isolated domains of Argonaute, which provided minimal information on the spatial and functional relationships between domains, or from full-length prokaryotic Argonaute, which elucidated overall architecture but did so for a homologue whose biological function in its respective organism was unknown.[40]

Three recent studies report the crystal structures of full-length eukaryotic Argonaute from humans (HsAgo2)[41,42] and the budding yeast *Kluyveromyces polysporus* (KpAgo).[43] In agreement with previous studies, the new structures demonstrate that Argonaute is a bilobed protein with a multidomain conformation (Fig. 2). The architecture of eukaryotic Argonaute is similar to that of the prokaryotic protein, indicating high structural conservation across kingdoms. The guide RNA is anchored at each end and threads through the central cleft of the protein, interacting with every domain and loop. This extensive threading structurally stabilizes HsAgo2, as demonstrated by the resistance of the binary complex to limited proteolysis relative to free protein.[41]

The MID domain, which forms a lobe with the PIWI and N domains, anchors the 5′ end of the guide strand. Extensive contacts between the 5′ monophosphate, a biochemical feature of miRNAs, and multiple side chains within the MID domain define the position of the guide strand relative to the enzyme active site. As the seed sequence threads along a narrow groove adjacent to the MID domain, it is stabilized by numerous contacts between its phosphate backbone, including RNA-specific 2′ OH groups, and the protein. Nucleotides 2–6 of the guide adopt an A-form conformation that is largely sequence independent, demonstrated by the well-defined electron density observed even when heterogeneous small RNA populations are bound by HsAgo2 or KpAgo in crystallographic preparations.[42,43] Bases within the seed are solvent exposed and, therefore, accessible for base pairing with a target. However, in HsAgo2, the stacked base pairing within the seed is interrupted by a kink between nucleotides 6 and 7 while, in KpAgo, the bases within the seed are tilted away from an orientation optimal for base pairing. These structural features suggest a requirement for conformational changes to the protein upon nucleation of pairing with a target. In HsAgo2, a second kink is formed beyond the seed between nucleotides 9 and 10 as the guide RNA threads into the protein. The 3′ end of the guide is anchored in the PAZ domain, which forms the second lobe of Argonaute.

While the structures of HsAgo2 and KpAgo include a guide RNA, they lack the target strand. Instead, insight into ternary complexes has been obtained from crystals of a full-length catalytically inactive mutant of *Thermus thermophilus* Argonaute (TtAgo) bound to a 5′ phosphorylated 21-nucleotide guide DNA with or without target RNAs.[44] As observed for HsAgo2 and KpAgo, the guide DNA in a binary complex with TtAgo adopts an A-form conformation with the 5′ and 3′ ends anchored in the MID and PAZ

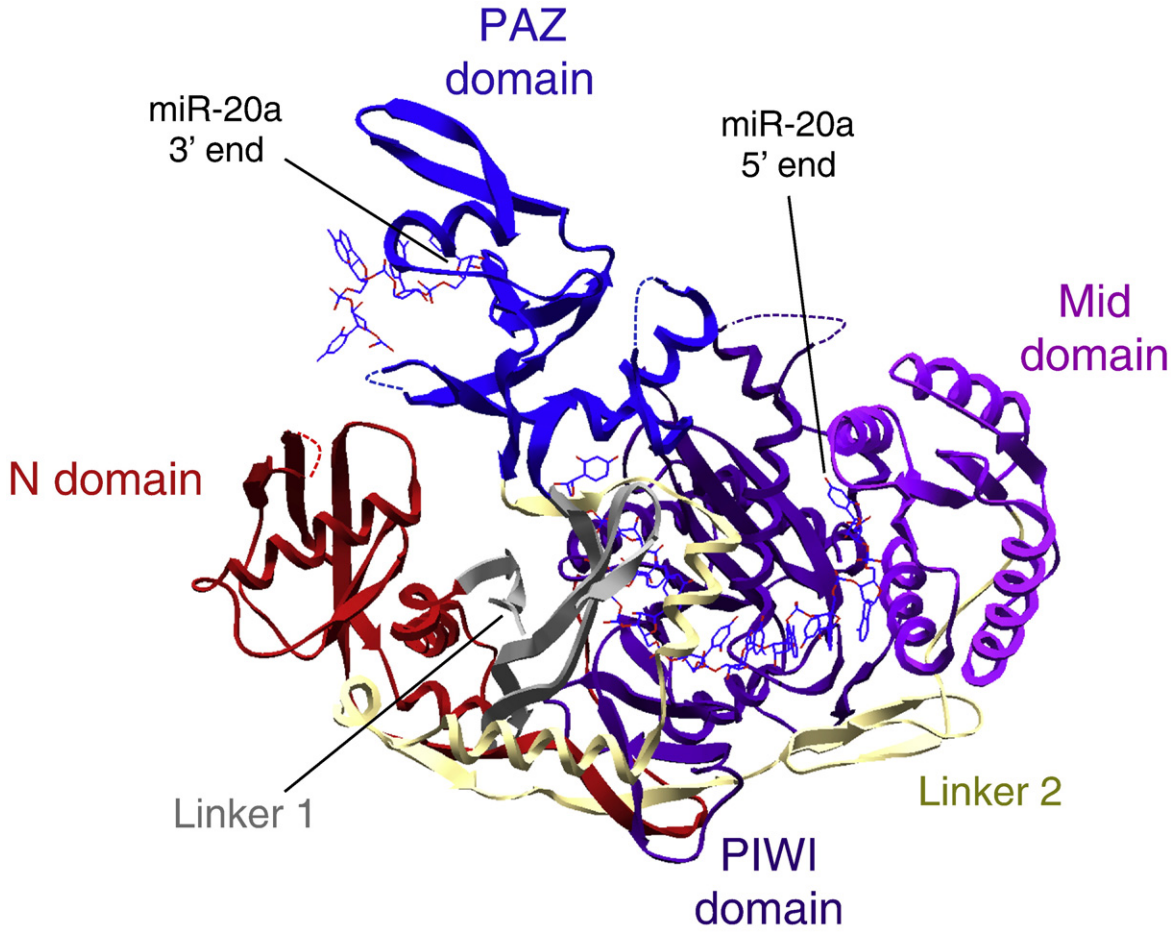

Fig. 2. Crystal structure of human Ago2 in complex with miR-20a. Ago2 is a bilobed protein with a multidomain conformation. The guide RNA is anchored at the ends by each lobe, with the MID domain binding the 5′ end and the PAZ domain binding the 3′ end. Bases within the seed of the guide strand are solvent exposed, with a kink between nucleotides 6 and 7. The crystal structure shown (Protein Data Bank ID: 4F3T) was reported by Elkayam *et al.*[41]

domains, respectively. Upon binding a target RNA, TtAgo undergoes a conformational shift through pivot-like domain movements that release the 3′ end of the guide strand from the PAZ pocket while maintaining the DNA–RNA duplex in an A-form helix maximally spanning positions 2–16 of the guide. This conformational shift positions two Mg^{2+} cations and three catalytic aspartate residues within the PIWI domain, which resembles RNase H in structure, for cleavage of the target RNA.

Although the catalytic activity of Argonaute has been ascribed to a catalytic triad ("DDX", where "X" is either aspartate or histidine) as described in TtAgo, RNase H is known to possess a "DEDD" catalytic tetrad. Indeed, the characterization of KpAgo identified a fourth conserved residue, glutamate, in the catalytic site.[43] Upon loading of the RNA duplex into KpAgo, the 3′ end of the guide strand is released from the PAZ while the glutamate completing the catalytic tetrad is inserted into the catalytic pocket to form a "plugged-in" conformation that promotes cleavage and subsequent release of the passenger strand. KpAgo bound to guide strand retains this plugged-in conformation and is thus primed for cleavage of additional substrates. This glutamate is required for RNAi *in vivo* in yeast, demonstrating biological activity.[43] The residue is also present in HsAgo2, where it is positioned within the active site, suggesting the conservation of a catalytic tetrad in multiple species.[42,43]

The new studies characterizing full-length eukaryotic Argonaute integrate the previous fragmentary glimpses of Argonaute structure into a complete picture of the binary complex and, in so doing, deepen insight into the activity of this class of enzymes. These studies also complement the structural analyses, reviewed in detail elsewhere,[45] of other conserved RNAi components such as Dicer. The remarkable structural conservation of Argonaute proteins across three kingdoms of life indicates an ancient function for short regulatory nucleic acids. Importantly, the new structures also raise additional questions, particularly about the unexpectedly kinked trajectory of the guide strand and the nature of the second state of eukaryotic Argonaute upon formation of a ternary complex with target RNA.

The Phenotypes of miRNAs

The biological function of miRNAs has been characterized at both the cellular and organismal levels. Sequencing of miRNAs from cell lines or whole tissues under a variety of treatment conditions, such as stress, has identified specific cell types or pathways associated with each miRNA seed family.[46] Furthermore, miRNA targets have been characterized through *in vitro* cell culture studies by at least two different approaches: (1) the identification and functional validation of targets through the use of prediction algorithms such as TargetScan or miRanda and (2) unbiased identification of miRNA-responsive genes by combining miRNA overexpression or inhibition experiments with genome-wide assays such as microarrays and, more recently, mRNA sequencing. Organismal functional studies have typically been carried out with loss-of-function studies using either germline or conditional deletion of miRNA genes. In Table 1, we summarize the cell-type specificity and tissue specificity of well-studied miRNAs, as well as their archetypal targets and relevance to disease. For the remainder of the review, we will focus on the four best-characterized of these miRNAs.

miRNAs participate in various circuit motifs with other regulators of gene expression, such as transcription factors.[91–93] At least two different network motifs have been identified within these circuits. In the first motif, termed coherent feedforward, miRNAs and the transcription factors that regulate them carry out the same activity on targets, namely, coordinated repression. In so doing, each factor reinforces the activity of the other. In the second motif, termed incoherent feedforward, the miRNA and transcription factor carry out opposing functions, allowing precise modulation of the temporal dynamics of gene expression to reduce noise and confer stability. Both motifs enable biological properties critical for phenotypic robustness (resistance to fluctuations in environment).[93,94] Generally, regulators of miRNAs are poorly understood and the annotation of miRNA promoters is incomplete. However, for several examples, the relationship between a miRNA and its regulator has been characterized at both the cellular and organismal level. Below, we trace the activity of four well-studied miRNAs.

miR-290~295 and the core pluripotency transcription factors

Pluripotent embryonic stem cells (ESCs) progress from a naïve state, in the inner cell mass of a pre-implantation embryo, to a primed state in the epiblast of a post-implantation embryo.[95] Subsequently, the cells of the epiblast undergo gastrulation to form the three germ layers, namely, mesoderm, ectoderm, and endoderm. Total loss of miRNAs, for example, through loss of Dicer, results in early embryonic lethality prior to gastrulation. Dicer- or DGCR8-null ESCs, which have been characterized *in vitro*, are unable to inactivate self-renewal programs or initiate differentiation into the three germ layers, further demonstrating that miRNAs are critical to early development.[96]

In naïve ESCs, the miR-290~295 family of miRNAs, schematized in Fig. 3a, comprises ~70% of all seed families.[97,98] This family is homologous to

Table 1. Tissue/cell type-specific miRNAs

miRNA family/cluster	Tissue/cell type	Target mRNAs	Target processes	Oncogene/tumor suppressor	References
let-7	Ubiquitous	Lin-28, Hmga2, Igf2bp1	Development, proliferation, organismal growth	Tumor suppressor	36,38,47,48
miR-1~133	Cardiac and skeletal muscle	Hand2, Irx5, Ptbp1, Ptbp2	Cell cycle, cardiac differentiation, splicing	Tumor suppressor	22,49
miR-15/16	Ubiquitous	Bcl2, Cyclin D, Cyclin E	Apoptosis, cell cycle	Tumor suppressor	50–52
miR-17~92	Ubiquitous/enriched in B cells	c-Myc, E2F, Bim	Proliferation, apoptosis	Oncogene	53–56
miR-22	Ubiquitous/enriched in cardiac and skeletal muscle	Purb	Calcium homeostasis, stress response	Tumor suppressor	57,58
miR-34	Ubiquitous/enriched in testis, brain, and lung	Sirt1, Snail, PNUTS	p53 pathway, EMT	Tumor suppressor	59–69
miR-122	Liver	AldoA, Hfe2	Cholesterol biosynthesis, lipid metabolism	Tumor suppressor	70,71
miR-124	Neurons	Ptbp1	Differentiation	Tumor suppressor	72
miR-143~145	Ubiquitous/enriched in smooth muscle	Klf4, Elk-1, myocardin	Differentiation	Tumor suppressor	73–76
miR-181	Immune cells	Shp1, Shp2, Dusp6, Tcl1	Differentiation, TCR signaling	Unknown	77–79
miR-193b~365	Brown adipocytes	Runx1t1	Differentiation	Unknown	80
miR-200	Epithelial tissue; olfactory bulb	Zeb1, Zeb2	EMT	Context-specific oncogene or tumor suppressor	81–83
miR-203	Epidermis	p63	Cell cycle, proliferation, differentiation	Tumor suppressor	84
miR-223	Myeloid cells	Mef2c	Differentiation	Tumor suppressor	85
miR-290~295	ESCs	Lats2, Rbl2, p21, Casp2	Cell cycle, proliferation, apoptosis, differentiation	Oncogene	86–89
miR-451	Erythrocytes	14-3-3z	Oxidant stress	Tumor suppressor	90

human miR-371~373, a cluster expressed in human ESCs. Although members of miR-290~295 are largely specific to ESCs, a notable exception is miR-293, which exhibits a distinct expression pattern and possesses a different, but related, seed sequence[99] (Fig. 3b). As naïve ESCs progress to the primed state, they downregulate miR-290~295 and activate miR-302~367,[100] a cluster conserved in both mouse and humans and whose members are related to the miR-290 family through a 6mer seed sequence (Fig. 3b). Subsequently, as the embryo develops further and differentiation progresses, expression of the miR-302~367 cluster turns off.[100]

miR-290~295 and miR-302~367 repress genes central to the self-renewal properties of ESCs (Fig. 3c). Specifically, miR-290~295 targets regulators of cell cycle and proliferation, such as p21 and Lats2;[86] apoptosis, such as caspase-2;[87] and DNA methylation, such as Rbl2, a transcriptional regulator of DNA methyltransferases.[88,89] The epiblast-expressed miR-302~367 represses Lefty1 and Lefty2, subtypes of transforming growth factor beta (TGFβ) ligands that regulate specification of body axes and specification of embryonic germ layers.[100] Accordingly, expression or inhibition of miR-302 respectively promotes or hinders the formation of mesendodermal lineages.[100] Supporting a role for these ESC-specific clusters in regulating pluripotency, several studies have reported that miR-290~295, its human homologue miR-371~373, and miR-302~367 enhance reprogramming of somatic cells into an induced pluripotent state.[101–103] In total, these miRNA clusters are centrally positioned in pluripotency networks and regulate gene expression programs that control the proliferation, survival, and self-renewal of ESCs.

Promoters of miRNA genes in ESCs have been identified by ChIP-sequencing (ChIP-seq) of trimethylated histone H3 lysine 4 (H3K4me3), a histone mark associated with the transcriptional start sites of most genes.[104] The promoters of miR-290~295 and miR-302~367 are occupied by Oct4, Sox2, Nanog, and Tcf3, which are core transcriptional regulators in ESCs.[104] An overlap of the transcriptional circuitry of ESCs with a list of mRNAs repressed by ESC-specific miRNAs suggests a role for these clusters in fine-tuning expression of pluripotency and differentiation programs.[105] For example, the genes Lefty1 and Lefty2, described above, are induced by the core embryonic transcription factors, such as Oct4, an illustrative example of an incoherent feedforward loop in the embryo.[104] Additionally, both the core pluripotency factors and the transcriptionally repressive Polycomb complex co-occupy promoters of miRNAs that are off in ESCs but will become activated in differentiated lineages, suggesting that these promoters are poised for activation.[105]

To examine the importance of ESC-specific miRNAs in development, germline knockouts

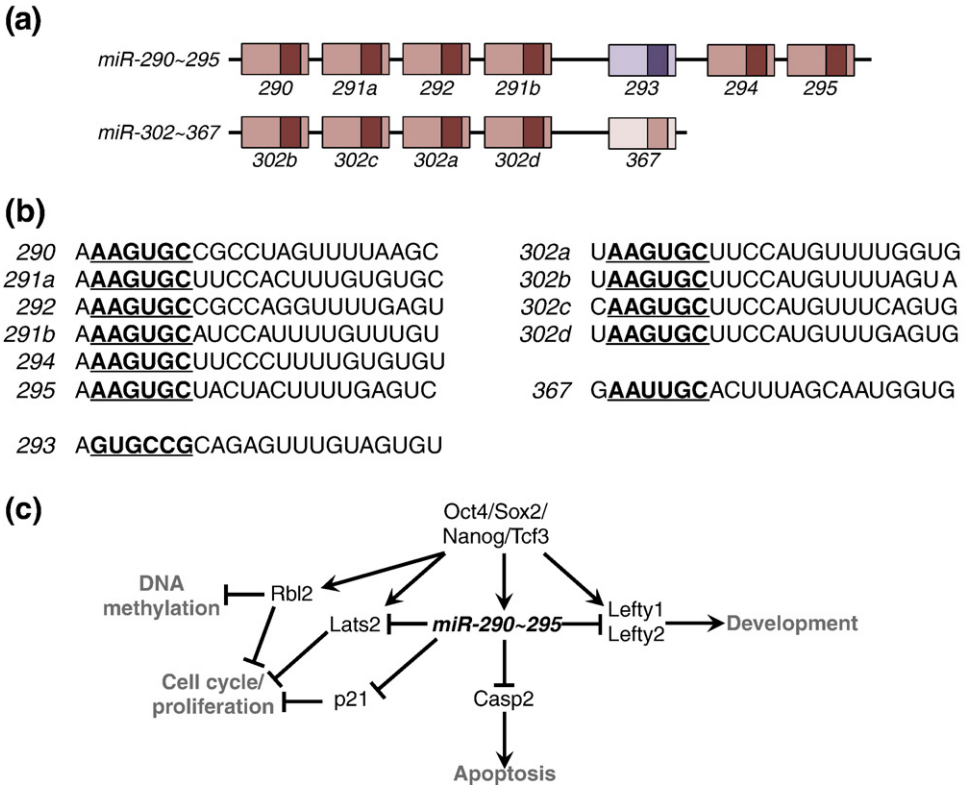

Fig. 3. ESC-specific miRNAs. (a) Gene structure of the murine miR-290~295 and miR-302~367 clusters. The pre-miRNA sequences are indicated as boxes, with mature miRNA sequences denoted in darker shades. Related family members are indicated by color. (b) Sequences of miRNAs. Each miRNA is grouped based on seed relationship. Seeds are boldfaced and underlined. (c) Summary of the miR-290~295 network.

(KOs) of miR-290~295 have been characterized and provide an interesting comparison to the Dicer KO animal as well as to *in vitro* studies investigating this cluster.[106] miR-290~295 KO animals exhibit defective migration and development of germ cells in the embryo, resulting in depletion of these cells in the adult. Males eventually recover from this early loss of germ cell count and are therefore fertile.[106] In contrast, adult females remain sterile. Loss of miR-290~295 also results in a partially penetrant embryonic lethal phenotype due to two distinct, abnormal phenotypes.[106] A subset of miR-290~295 KO embryos localize outside of the yolk sac, a phenotype that may reflect the expression and importance of this cluster in the trophectoderm, which develops into the placenta. Additionally, some miR-290~295 KOs exhibit developmental delays, including reduced somite numbers and defects in neural tube closure. Therefore, while miR-290~295 is important for normal embryonic development, it is not absolutely required for ESC differentiation or development into adulthood.

The complexity of the phenotype of miR-290~295 KO animals highlights several key properties of miRNAs. The incomplete penetrance of the phenotype suggests a role for this cluster in maintaining robustness, with stochastic variations in local environment possibly leading to defects in a subset of embryos. This model is in line with observations indicating that miR-290~295 participates with embryonic transcription factors in both coherent and incoherent feedforward loops, possibly to regulate the kinetics of gene expression during differentiation.[104] Alternatively, the incomplete penetrance may be explained by functional compensation by other miRNAs, derived from miR-302~367 or, alternatively, from the Sfmbt2 cluster, which possesses related seed sequences but is located in a different locus.[107,108] The expression of miR-293, whose seed sequence and expression pattern differ from the other members of its cluster, also raises the possibility that the phenotypes observed in miR-290~295 KO embryos *versus* adults are driven by distinct miRNA seed families.[99] These possibilities highlight many of the challenges, and opportunities, in the study of miRNA function.

Let-7 and growth

Let-7 is one of the first miRNAs to be discovered and, as reviewed extensively elsewhere,[109,110] was identified in a genetic screen in nematode for

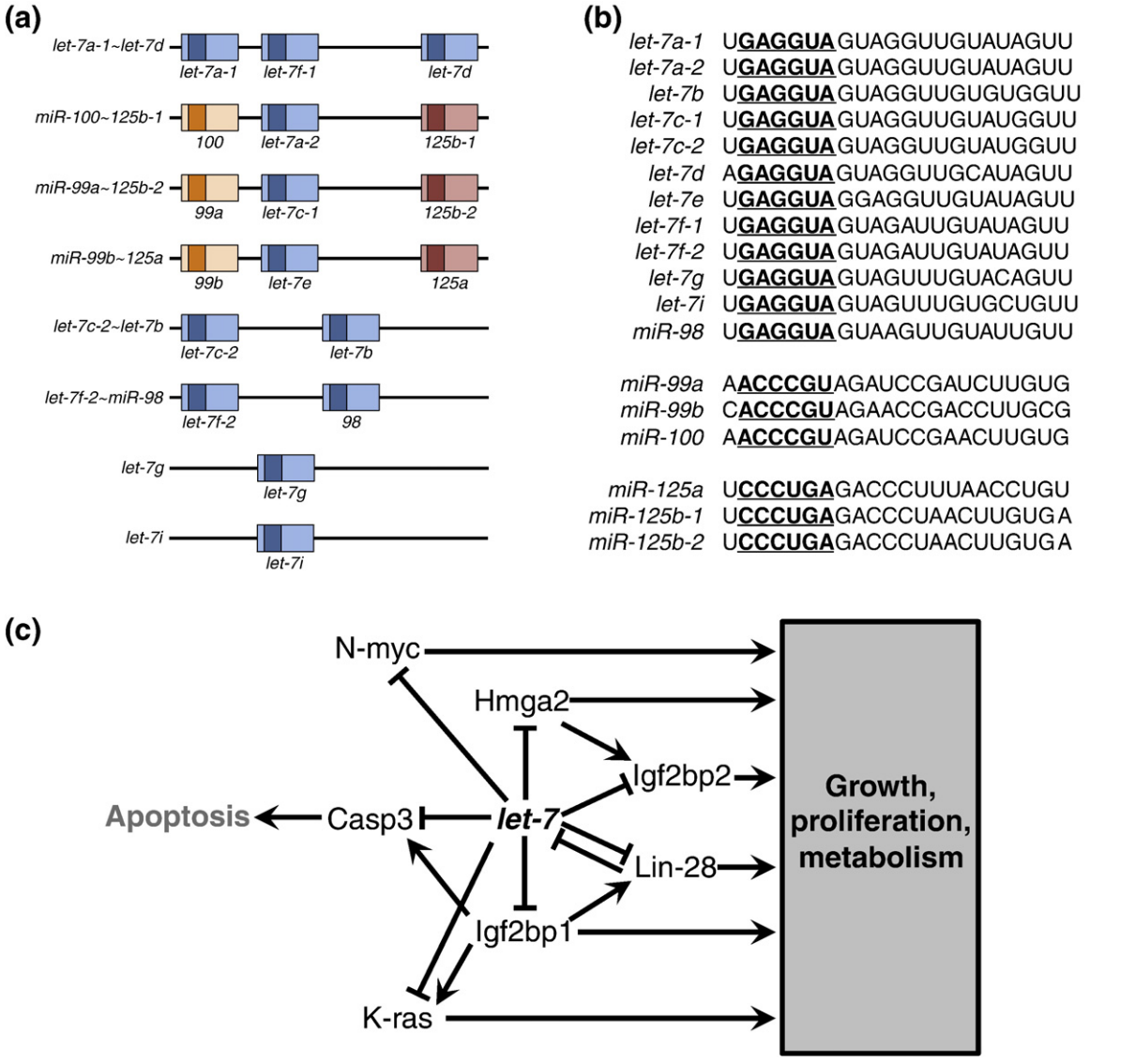

Fig. 4. The let-7 genes. (a) Gene structure of the let-7 genes. At three loci, let-7 is clustered with the miR-99/100 and miR-125 families. The pre-miRNA sequences are indicated as boxes, with mature miRNA sequences denoted in darker shades. Related family members are indicated by color. (b) Sequences of miRNAs. Each miRNA is grouped based on seed relationship. Seeds are boldfaced and underlined. (c) Summary of the let-7 network. Let-7 genes are characterized by a shared role in regulating proliferative and metabolic pathways activated in the embryo. Let-7 targets are densely interconnected and regulate one another.

regulators of developmental timing. *C. elegans* encodes several let-7 family members, namely, let-7, miR-48, miR-84, and miR-241. Loss of the let-7 family in nematode results in the reiteration of developmental larval stage events. This phenotype is a result of the upregulation of multiple let-7 targets, including lin-28, an RNA-binding protein, and daf-12, a member of a nuclear hormone receptor superfamily. These two genes also feed back to regulate let-7: lin-28 binds pre-let-7 and inhibits its maturation, while daf-12 transcriptionally activates or represses let-7 expression in the presence or absence, respectively, of its ligand, dafachronic acid.[111,112]

Let-7 is highly conserved across species, including mammals, and is expressed broadly across tissue types.[110] The number of loci encoding let-7 has expanded to eight in mouse and humans (Fig. 4a). At three loci, let-7 is clustered with the miR-99/100 and miR-125 families, which possess distinct seed sequences (Fig. 4b). In mammals, as in worm, expression of mature let-7 is activated during development and maintained in the adult.[113] Although primary transcripts of let-7 are expressed in ESCs, precursor and mature let-7 are undetectable, indicating a block at the level of processing.[114] During early development, maturation of pre-let-7 is

inhibited by Lin-28a and Lin-28b,[47,115,116] homologues of nematode lin-28 whose functions are described in greater detail below. As in worm, the mammalian Lin-28 genes are targets of let-7, thus constituting a conserved negative feedback loop.[116] In many adult tissues, let-7 is expressed abundantly and functions as a tumor suppressor.

Many mammalian let-7 targets have been identified and are strongly characterized by their roles in growth, metabolism, and development (Fig. 4c). The best characterized of these targets are Lin-28, Hmga2, and the Igf2bp1–3 family. These genes constitute in its entirety a class of genes termed "oncofetal" because of their expression in the embryo, inactivation in most adult tissue, and reactivation in tumors. Of these, the mammalian paralogues Lin-28a and Lin-28b are best understood and represent quintessential oncofetal genes. Lin-28a/b regulate organismal growth and metabolism.[117] Transgenic mice overexpressing Lin-28a exhibit increased body size and delayed onset of puberty.[48] Additionally, transgenic mice overexpressing either Lin-28a or LIN28B exhibit an altered metabolism, manifested as increased insulin sensitivity through activation of the insulin–PI3K–mTOR pathway.[118] In humans, polymorphisms in *LIN28B* have been associated with variations in height and the timing of menarche.[119–123] Demonstrating a role for mammalian Lin-28 in maintaining "stemness", overexpression of Lin-28, in combination with Oct4, Sox2, and Nanog, promotes induction of pluripotency in somatic fibroblasts.[124] Inhibition of let-7 by overexpression of Lin-28b in adult hematopoietic stem cells results in the reprogramming of these cells into a fetal state.[125] Consistent with a role in proliferation and growth, mammalian Lin-28 genes are commonly activated in tumors.[126] LIN28B induces neuroblastoma in patients by suppressing let-7 and enhancing expression of MYCN, another let-7 target.[127] In total, the Lin-28 genes regulate proliferative and metabolic pathways at least in part through their modulation of let-7.

Another well-characterized oncofetal let-7 target is Hmga2, a non-histone chromatin factor. Hmga2 is normally expressed in the embryo and is off in most adult tissues. Knockout of Hmga2 in mouse leads to a dwarf phenotype in which mutant animals are smaller than wild-type littermates.[128] Constitutive overexpression of transgenic Hmga2 in mouse leads to increased organismal size and changes in composition of body fat,[129,130] a phenotype very similar to that observed for Lin-28 transgenic mice. In humans, genome-wide association studies have linked polymorphisms in *HMGA2* to variations in human height and predisposition to diabetes.[131,132] Hmga2 is also oncogenic.[133] It is often translocated in benign lipomas and salivary gland tumors, leading to fusion of its AT-hook DNA-binding domains to a translocation partner.[134] Expression of Hmga2 is observed in high-grade tumors in various cancers, such as ovarian cancer, and associated with poor patient prognosis.[135] Experimental evidence supports a causal role for Hmga2 in tumorigenesis. Transgenic mice overexpressing Hmga2 develop benign lipomas and other mesenchymal tumors, as well as pituitary adenomas.[129,130] Finally, changes in the 3′UTR of Hmga2 mRNA, for example, by mutation, promote resistance to let-7-mediated repression and subsequent cellular growth.[36]

The Igf2bp1–3 family of RNA-binding proteins is yet another set of oncofetal let-7 targets that share many of the properties of Lin-28 and Hmga2. Knockout of Igf2bp1 results in a dwarf phenotype,[136] while its transgenic overexpression in mice leads to tumor development.[137] Overexpression of Igf2bp1 *in vitro* promotes anchorage-independent growth.[37] As with Hmga2, alterations in the 3′UTR of Igf2bp1, for example, through the use of alternative polyadenylation sites, alter its sensitivity to repression by let-7.[37] Igf2bp2 and Igf2bp3 are less well characterized but are likely integrated into this let-7-regulated network of growth and metabolism. Members of the Igf2bp family are associated with tumors and are strongly correlated with each other as well as with Hmga2.[38,138] Demonstrating the dense interconnections in this pathway, Igf2bp1 induces expression of Lin-28b and the oncogene K-ras, another let-7 target.[139,140] Additionally, Hmga2 directly induces transcription of Igf2bp2 during regeneration of muscle.[141] Paradoxically, let-7 also represses Caspase-3,[142] an activator of apoptosis that is also induced by Igf2bp1.[140] This pro-survival activity of let-7 is poorly understood but may reflect a role for this miRNA in balancing two related but opposing pathways, consistent with published models proposing that miRNAs regulate the dynamics of state transitions.

There are also intriguing links between targets of let-7, germ cell development, and life span. In fruit fly, an axis between let-7 and Imp (the fruit fly orthologue of the Igf2bp family) regulates aging of the testis stem cell niche.[143] In the testis, Imp stabilizes the RNA-binding protein Upd, which promotes germ line stem cell self-renewal. As the organism ages, let-7 downregulates Imp, resulting in a reduction in Upd levels, loss of self-renewal, and depletion of germ line stem cells, thus demonstrating a negative correlation between let-7 levels and germ cell proliferation. In nematode, let-7, its transcriptional activator daf-12, and its target lin-14 integrate signals from the gonad to regulate life span.[144] When the *C. elegans* germ line is removed, there is an increase in expression of the let-7-related miRNAs, miR-84 and miR-241, resulting in the suppression of lin-14 and akt and the subsequent stimulation of FOXO signaling. This cascade ultimately leads to an increase in life span. Finally, in mammals, Lin-28 is required for primordial germ cell development.[145] Similarly, Hmga2-deficient male mice are infertile due to a lack of spermatozoa.[146]

These studies demonstrate a conserved role for let-7 targets in germ cell development. In total, the deeply conserved let-7 network appears to regulate the intimately linked processes of proliferation, growth, development, metabolism, and longevity.

miR-17~92 and c-Myc

miR-17~92, also known as oncomiR-1, is a widely studied, oncogenic cluster of miRNAs that encodes four different seed families and has two paralogues, namely, miR-106b~25 and miR-106a~363 (Fig. 5a and b). miRNAs derived from miR-17–92 and miR-106b~25 are broadly expressed during development, including in ESCs and midgestation embryos, and across adult tissues, including liver, heart, and brain.[53] miR-17~92 is critical to development. Patients with hemizygous germline deletion of miR-17~92 develop Feingold syndrome, previously associated only with mutations in MYCN, a proliferative gene and let-7 target, as described above. This disorder is characterized by microcephaly, short stature, and digital abnormalities.[147] miR-17~92 also regulates proliferation and is often amplified or overexpressed in various tumor types, including B cell lymphomas and small cell lung carcinoma, and drives tumorigenesis in mouse models of lymphoma and leukemia.[148–151]

The miR-17~92 cluster participates in a circuit with c-Myc and E2F[54–56,152] (Fig. 5c). c-Myc transcriptionally induces miR-17~92 and the transcription factors E2F1,[54] E2F2, and E2F3.[56] E2F1–3 also induce transcription of miR-17~92.[54–56] miR-17 and miR-20, members of the same seed family within the miR-17~92 cluster, in turn repress translation of E2F1–3, exemplifying negative feedback in the case of E2F regulation[54–56] and an incoherent feedforward loop in the case of c-Myc.[54] Consistent with these functional relationships, miR-17~92 cooperates with c-Myc to induce murine lymphoma.[148] Additionally, transcription of miR-17~92 is regulated by BMP signaling in the heart.[153,154] Many additional pathways, summarized recently,[149] are regulated by miR-17~92: proliferation (Cyclin D1[155] and p21[39,156]), TGFβ signaling (TGFβ-R2, SMAD2, and SMAD4[157]), survival (PTEN,[158,159] BIM,[53,160] and Fas[161]), and cell-type-specific processes such as differentiation (CEBPA[162] and GATA6[162]). Interestingly, the seed sequence of the miR-17 family (AAAGUGC) overlaps with the seed of the ESC-specific miR-290 family (AAGUGCU). Both families

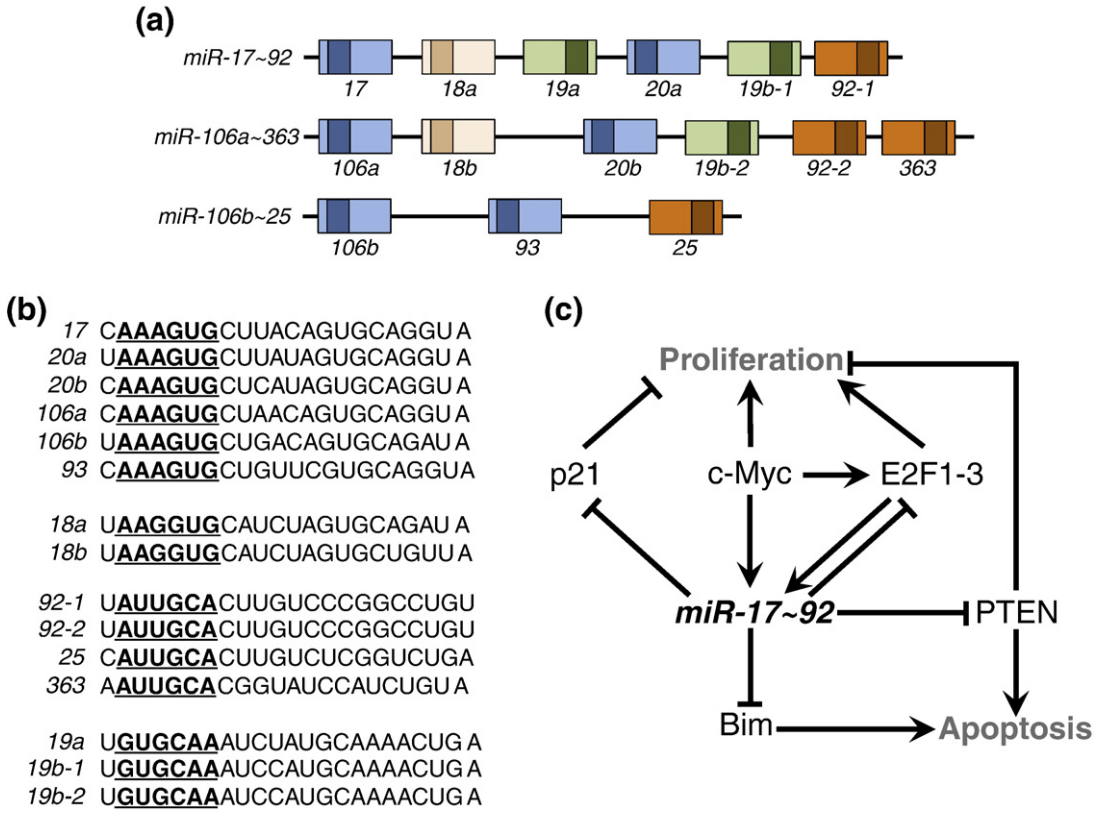

Fig. 5. The miR-17~92 genes. (a) Gene structure of paralogues miR-17~92, miR-106a~363, and miR-106b~25. The pre-miRNA sequences are indicated as boxes, with mature miRNA sequences denoted in darker shades. Related family members are indicated by color. (b) Sequences of miRNAs. Each miRNA is grouped based on seed relationship. Seeds are boldfaced and underlined. (c) Summary of the miR-17~92 network.

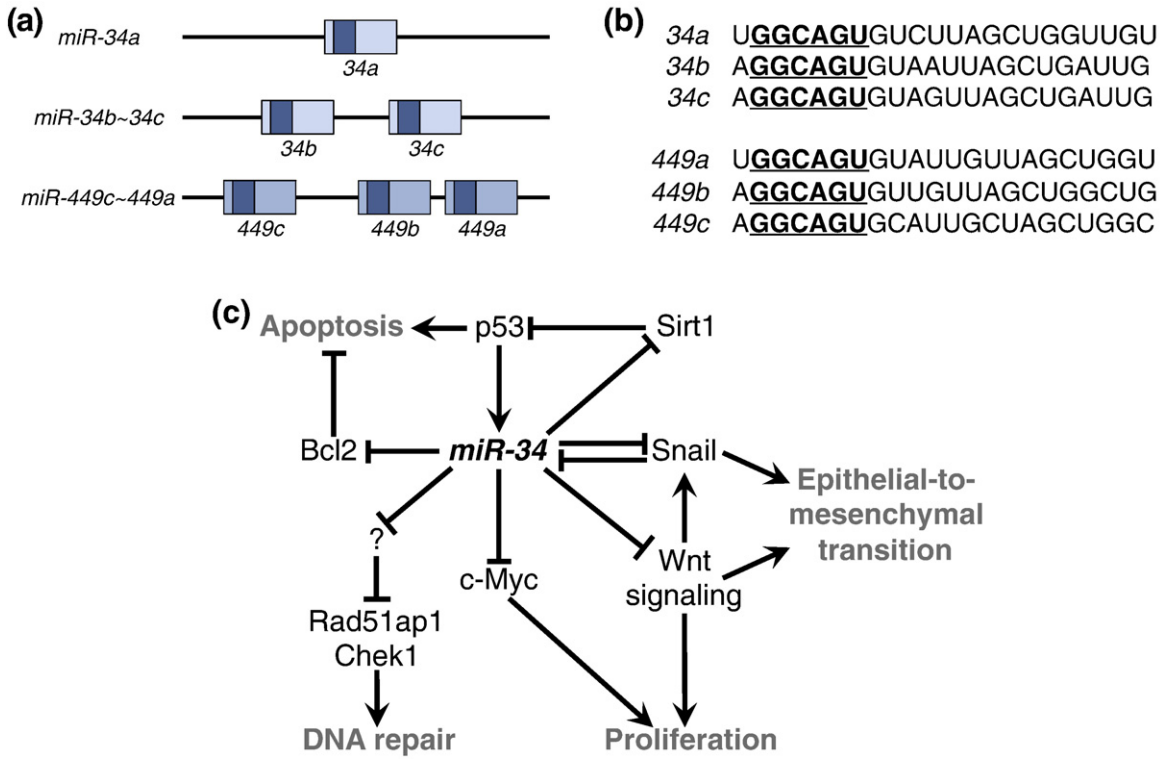

Fig. 6. The miR-34 and miR-449 genes. (a) Gene structure of miR-34a, miR-34b~34c, and miR-449c~449a. The pre-miRNA sequences are indicated as boxes, with mature miRNA sequences denoted in darker shades. Related family members are indicated by color. miR-34 and miR-449 share the same seed sequence. (b) Sequences of miRNAs. Each miRNA is grouped based on seed relationship and sequence similarity. Seeds are boldfaced and underlined. (c) Summary of the miR-34 network.

repress common targets[154] that regulate proliferation, such as Lats-2 and p21, and differentiation, such as Lefty1, Lefty2, and TGFβ-R2. Furthermore, miR-17~92 was recently demonstrated to enhance induction of pluripotency.[163] These results suggest that miR-17~92 is a somatic counterpart to the miR-290 family that contributes to the de-differentiation and plasticity of tumors.

A mouse model of miR-17~92 loss has been generated.[53] Germline deletion of miR-17~92 results in early postnatal lethality, while animals with germline deletion of either miR-106b~25 or miR-106a~363 are viable and fertile. Triple KO of all paralogues, or double KO of miR-17~92 and miR-106b~25, results in embryonic lethality by E15,[53] indicating at least partially redundant functions. miR-17~92 KO animals exhibit several prominent developmental abnormalities: (1) skeletal defects, which phenocopy the symptoms observed in patients with germline mutations in this cluster;[147] (2) lung hypoplasia;[53] (3) ventricular septal defect;[53] and (4) a failure in fetal B cell development.[53] miR-17~92 is also required for adult B cell development. Transplant of miR-17~92-deficient hematopoietic cells fails to reconstitute hematopoiesis in lethally irradiated mice,[53] while tissue-specific deletion of Dicer in B cell progenitors leads to increased apoptosis.[160] These immunological defects are partially due to de-repression, in pro-B cells, of Bim, a pro-apoptotic gene and target of miRNAs clustered in miR-17~92.[53,160] In myeloid cells, the miR-17 family promotes proliferation by targeting sequestome 1, a ubiquitin-binding protein that regulates autophagy-mediated protein degradation.[164] In total, miR-17~92 plays an important role in various tissue types, consistent with its broad expression pattern.

In addition to elucidating the function of this cluster in normal development and physiology, mouse models of miR-17~92 function have been used to characterize the role of this cluster in promoting tumorigenesis. In an Eμ-Myc model of murine lymphoma, deletion of miR-17~92 results in reduced lymphoma burden due to increased apoptosis of tumor cells.[158] Overexpression of miR-19, a distinct seed family within miR-17~92, is necessary and sufficient to promote c-Myc-induced tumorigenesis and rescues the phenotype of miR-17~92 deletion.[158,159] The activity of miR-19 is mediated by its downregulation of the tumor suppressor PTEN.[158,159] A second mouse model of cancer, specifically retinoblastoma, also

demonstrates that miR-17~92 participates in the development of tumors.[165,166] While overexpression of miR-17~92 alone does not induce tumors, combining this transgene with mutations in the Rb pathway promotes the formation and metastasis of retinoblastoma.[165] In contrast to the Eμ-Myc model, in which miR-19 represses apoptosis, seed-family-specific inhibition of miRNAs with antagomirs suggests that the activity of this cluster in retinoblastoma is independent of miR-19 as well as apoptosis, instead promoting proliferation through repression of p21 by the miR-17 family.[165] In complementary loss-of-function studies, deletion of miR-17~92 in retinal progenitor cells in the context of combined Rb and p53 loss results in suppression of retinoblastoma, a result again attributable primarily to the miR-17 family.[166] Thus, various family members within the miR-17~92 cluster possess oncogenic activities.

miR-17~92 exemplifies many of the properties of miRNAs relevant to the regulation of gene expression networks. This cluster contains multiple miRNA seed families whose members are expressed from multiple paralogous loci. Clustered, unrelated seed family members, such as miR-17 and miR-19, regulate distinct but related biological functions, namely, proliferation and apoptosis, respectively. In normal tissues and under pathological conditions, miR-17~92 promotes growth by targeting genes that participate in common pathways, including the c-Myc/Rb/E2F axis and its targets. One of the many outstanding questions in the field concerns the tissue-specific phenotypes of the KO animals. For example, do the lung and cardiac defects result from misregulation of the same developmental and proliferative axes observed in blood or are distinct pathways responsible for these phenotypes? What are the functions of the additional family members expressed from these clusters? The experimental tools currently available, such as miR-17~92-conditional mice, tissue-specific Cre transgenes, and miRNA expression constructs, may be sufficient to answer these questions.

miR-34 and the p53 response

p53 is a commonly mutated tumor suppressor that coordinates multiple pathways, ranging from damage repair to cell death, to counter stress.[167] Given its profound biological and clinical importance, the discovery that p53 induced the expression of a miRNA was met with great interest. However, follow-up studies have led to conflicting results that suggest a functional complexity not predicted by current models.

Upon exposure to stress, p53 induces the miR-34 family of miRNAs in murine embryonic fibroblasts,[59] a mesenchymal cell type, and HCT116 colon cancer cells,[60] an epithelial cell type. Three miRNAs comprise the miR-34 family (Fig. 6a and b): miR-34a is expressed as a single miRNA from an intergenic locus, while miR-34b and miR-34c are expressed as an intergenic cluster. The promoters of both loci possess p53 binding sites that are bound and activated by p53. Therefore, these miRNAs are direct transcriptional targets of p53.[59–64] Additionally, the miR-449 family, encoded as a cluster of three miRNAs in a single locus, shares a seed sequence with miR-34 and therefore belongs to the same seed family.[65] However, miR-449 and miR-34 are divergent in sequence outside of the seed region (Fig. 6b). The p53 responsiveness of miR-449 has not been systematically characterized.

Supporting a role in the p53 pathway, exogenous expression of miR-34 mediates anti-proliferative effects, p53-mediated apoptosis,[62] and senescence.[59] Multiple strategies, including mRNA expression profiling,[60] proteomics,[168] and capture of miRNA--bound mRNAs,[169] have identified targets of miR-34 in these pathways[170] (Fig. 6c). miR-34 participates in a positive feedback loop with its transcriptional activator by repressing SIRT1, a gene responsible for deacetylation, and subsequently reduced activity, of p53.[66,171] Additional targets of miR-34[60,170] include regulators of cell cycle, such as CDK4[59,172] and Cyclin E;[59,64] apoptosis, such as Bcl2 and DcR3;[64] and proliferation, such as c-Myc.[173] miR-34 also regulates multiple genes in the DNA damage pathway.[169] This activity includes induction of DNA repair genes, such as Rad51ap1 and Chek1,[60] presumably through incompletely characterized intermediate targets. Consistent with a common functional role, both p53 and miR-34 pose a barrier to the reprogramming of somatic cells into induced pluripotent stem cells, although this activity may be due solely to the regulation of proliferation.[174] Additionally, gene expression analyses followed up with functional experiments have demonstrated a role for miR-34 in downregulating multiple components in canonical Wnt-signaling, a pathway important in development, epithelial-to-mesenchymal transition (EMT), and tumorigenesis.[175] SNAIL, a transcription factor regulated by Wnt-signaling, participates in a double-negative feedback loop with miR-34 to form a bistable switch that regulates EMT.[67,68] As a further illustration of the complexity and redundancy of miRNA-regulated pathways, p53 also induces the miR-200 family of miRNAs, which repress ZEB1 and ZEB2, transcriptional activators of EMT.[176] Clinically, multiple studies have reported reduction in miR-34 expression in a variety of tumor types, including lung and breast cancer,[170,176] and *in vivo* functional follow-ups, such as in the case of hepatocellular carcinoma, have confirmed a tumor-suppressive role for this miRNA in tumor-derived cell lines.[177]

While overexpression studies have demonstrated that miR-34 is sufficient to activate p53-related pathways, an elegant and exhaustive genetic

loss-of-function study in mouse suggests that miR-34 is not necessary for a canonical p53 response. Deletion of both miR-34 loci, and consequently all three miR-34 family members, in mouse has revealed a surprisingly mild phenotype.[65] The mutant animals develop normally, an observation consistent with the normal development of p53 KO mice. However, unlike p53 KO cells, miR-34 KO cells respond normally to genotoxic stress by activating cell cycle arrest or apoptosis in murine embryonic fibroblasts and thymocytes, respectively. Additionally, both wild-type and miR-34 KO animals are sensitive to gamma irradiation while, in contrast, p53 KO animals are resistant. miR-34 KO animals also do not form the spontaneous tumors that are a hallmark of both p53 heterozygosity and loss.[65] Furthermore, miR-34 is expressed in testes, lung, and brain independent of p53 expression, suggesting functions additional to the p53 pathway. The observation that miR-34 is expressed in brain supports recent reports demonstrating that this miRNA regulates development of the nervous system.[178,179] The miR-449 gene, a transcriptional target of E2F1 and regulator of cell cycle progression, encodes miRNAs that share a seed sequence with miR-34.[180] Since this gene is intact in miR-34 KO animals, it could in principle compensate for miR-34 loss. However, with the exception of testis, this miRNA is not appreciably expressed in the same tissues as miR-34. Additionally, miR-449 is not upregulated in miR-34 KO animals.[65] Nonetheless, a formal test of functional redundancy will require additional compound mutant mice.

Despite its strong transcriptional link to p53, miR-34 in the mouse appears to be dispensable for canonical p53 stress response, suggesting context-dependent functions for this miRNA family. Consistent with this possibility, a recent study demonstrated a role for miR-34 in promoting age-associated cardiomyocyte cell death through repression of PNUTS, a regulator of apoptosis and DNA damage response.[69] Additional analyses of miR-34 KO animals in various tumor models with greater numbers of mice may uncover tissue-specific roles for this miRNA in p53-mediated tumor suppression. Nonetheless, even in these initial studies, the findings contrast with reports that inhibition of miR-34 through complementary antagomirs compromises p53 response. These results may reflect a phenotypic difference between acute loss of miR-34 activity in the inhibition experiments and early developmental loss in the mouse model. The observation of a surprisingly mild mouse KO phenotype under basal, unstressed conditions is a common feature in miRNA studies. A notable example is miR-143~145, a cluster of two unrelated miRNAs implicated as tumor suppressors in leukemia and as inhibitors of pluripotency.[73–75] Mouse KOs of this cluster progress normally into adulthood without developing spontaneous tumors. However, these animals exhibit intestinal collapse due to defects in smooth muscle function. The discrepancies between *in vivo* and *in vitro* models raise the possibility that early loss of a miRNA family, for example, by deletion in the germline, leads to functional compensation mediated by the rewiring of miRNA-regulated networks. For many such miRNA KO animals, it will be important to determine if acute, tissue-specific deletion of conditional alleles yields phenotypes more consistent with those observed in cell culture through overexpression or, conversely, antagomir-mediated inhibition.

Concluding Remarks

Much progress has been made in understanding miRNA function. These small RNAs orchestrate the activities of functionally related genes within discrete and isolatable networks. As additional targets of miRNAs are delineated through global gene expression profiling and animal studies, the thus far binary sets of interactions that have been identified will be placed into a larger context, yielding greater insight not only into miRNA function but also into the circuitry of gene expression.

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Abbreviations used:
miRNA, microRNA; pre-miRNA, precursor miRNA; RISC, RNA-induced silencing complex; Ago2, Argonaute 2; UTR, untranslated region; ESC, embryonic stem cell; TGFβ, transforming growth factor beta; KO, knockout; EMT, epithelial-to-mesenchymal transition.

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