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Analysis of microRNA knockouts in mice

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MicroRNAs (miRNAs) are small noncoding RNAs that act as potent regulators of gene expression. The discovery of miRNAs with specific temporal and spatial expression patterns revealed a hidden layer of post-transcriptional gene regulation. Furthermore, differential expression of miRNAs during disease progression identified miRNAs as relevant candidate genes in human pathologies. Currently the exact roles of miRNAs in human development and disease progression remain largely unknown. There have been recent efforts to study the loss of these genes *in vivo* and this review will discuss published miRNA knockout mouse models, highlighting their potential mechanisms of action *in vivo*.

INTRODUCTION

MicroRNAs (miRNAs) are small noncoding RNAs of about 22 nucleotides (nt), which can bind to target RNAs based on sequence complementarity and direct post-transcriptional regulation of gene expression. First discovered in 1993 by genetic screens in *Caenorhabditis elegans*, the *lin-4* miRNA was treated as a unique phenomenon of nematode biology (1,2). The subsequent discovery of a second miRNA encoded by the *let-7* gene and its conserved temporal expression patterns in a wide range of animal species led us to recognize miRNAs as prevalent noncoding RNAs conserved among eukaryotes (3). Their diversity was confirmed using bioinformatics and subsequent cloning (4–6). With more than 500 confirmed miRNAs in mammals, they have rapidly emerged as a major class of regulatory genes that control development and disease processes in mammals (7–9). The physiological importance of individual miRNAs in mammals has only recently begun to emerge with the analysis of miRNA knockout mice, along with the discovery of mutations within miRNAs themselves or their target mRNAs (9–11). Here we discuss how miRNAs regulate a series of complex biological processes by reviewing published mouse knockout models with an emphasis on physiological and genetic evidence.

OVERVIEW OF miRNA BIOGENESIS

This large family of miRNA genes has great diversity in genomic organization. Examination of mammalian miRNAs revealed that about one-third are expressed from introns of known protein-coding genes, with the remainder found distant from previously annotated genes (7). Whereas intronic miRNAs can be transcribed simultaneously with their host genes, intergenic miRNAs can be expressed from independent transcription units (12,13). They are frequently found as polycistronic clusters that are coordinately expressed (14,15). Although RNA Pol II transcribes the majority of miRNAs, RNA Pol III-transcribed miRNAs have been found (16,17).

A canonical pathway generates most miRNAs, where sequential processing by RNases releases the mature ~22 nt miRNA from a longer transcript. The first processing step occurs in the nucleus, where the microprocessor complex recognizes a stem-loop structure in the primary miRNA (pri-miRNA) transcript. This complex includes the RNase III enzyme Drosha, which cleaves the miRNA-bearing stem-loop with the aid of other factors including DGCR-8 (18). The mature miRNA may reside on either arm of the stem-loop structure, and cleavage by Drosha establishes the 5′ or 3′ end of the mature miRNA. The resulting precursor miRNA (pre-miRNA) is actively transported into the cytosol via the karyopherin Exportin-5 (19,20). In the cytosol, pre-miRNAs are incorporated into a complex that contains a second RNase III enzyme, Dicer (21). With the help of TRBP and other factors, Dicer removes the loop from the pre-miRNA, leaving an RNA duplex (22). Typically one strand of the duplex is selected to become the mature miRNA (guide strand), whereas the other strand (star strand) is degraded quickly. After the mature miRNA is formed, it associates with an Argonaute (Ago) protein, the primary effectors of the RNA-induced silencing complex (RISC) (23). There are rare examples of miRNAs made by alternative pathways that are independent of processing by Drosha or Dicer (24–26).

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Table 1. miRNA knockout in mice

Gene	Expression	Phenotypes	Reference
miR-155	Thymus, spleen	Lung airway remodeling, defects in adaptive immunity	40
miR-155	Thymus, spleen	Impaired T-cell- and B-cell-dependent immunity	41
miR-17–92	Detectable in most adult tissues	100% postnatal death with cardiac and lung defects	47
miR-106a–363	Detectable in most adult tissues	No obvious phenotype	47
miR-106b–25	Detectable in most adult tissues	No obvious phenotype	47
miR-15a/16-1	Widely expressed	CLL-associated phenotypes at old age	67
miR-144/451	Erythroid lineage	Erythroid hyperplasia, splenomegaly, anemia	68
miR-451	Erythroid lineage	Similar to miR-144/451 mice	68
miR-150	Mature B and T cells	B1 cell expansion	71
miR-223	Myeloid cells	Expanded granulocyte lineage	73
miR-1-2	Heart, skeletal muscle	~50% lethality with cardiac defects	43
miR-208a	Heart	Reduced cardiac hypertrophy in response to stress	42
miR-208a	Heart	Minor cardiac conduction defects	91
miR-208b	Heart, skeletal muscle	No obvious phenotype	56
miR-499	Heart, skeletal muscle	No obvious phenotype	56
miR-133a-1	Heart, skeletal muscle	No obvious phenotype	53
miR-133a-2	Heart, skeletal muscle	No obvious phenotype	53
miR-206	Skeletal muscle	No obvious phenotype	78
mRi-126	Endothelial cells	~40% lethal with embryonic and perinatal hemorrhages	50
mRi-126	Endothelial cells	~50% embryonic death with hemorrhages	49
miR-143/145	Developing heart, adult SMCs	Reduced media thickness in aorta	64
miR-143/145	Developing heart, adult SMCs	Synthetic smooth muscle cells, reduced media thickness	65
miR-143/145	Developing heart, adult SMCs	Thinner smooth muscle layers in vessels	66
miR-143	Developing heart, adult SMCs	No obvious phenotype	66
miR-145	Developing heart, adult SMCs	Similar to miR-143/145 mice	66
miR-375	Islet	Hyperglycemia	83
miR-140	Cartilage	Short stature and aged-related osteoarthritis	86
miR-182	Broad expression, high in eye	No obvious phenotype	89

SMC, smooth muscle cells; CLL, chronic lymphocytic leukemia.

The molecular mechanism by which the RISC regulates target gene expression is an area of intense study (27). Mammalian miRNAs guide RISC to their targets by complementary base-pairing to nucleotides 2–8 of the miRNA (28). This short but critical region is known as the seed sequence, and miRNAs can be grouped into families based on shared seed sequences. Since the miRNA seed is only 7 nt long, each miRNA can potentially regulate hundreds of genes when considering the large number of possible binding sites in mRNAs. The first identified miRNA-binding sites were found in the 3′-UTR of target mRNAs, and prediction algorithms have largely focused on these regions when searching for potential miRNA targets (28–31). Recently, unbiased studies have identified miRNA-binding sites within the 5′-UTR and in the coding region (32). The task of identifying bona fide *in vivo* miRNA–mRNA interactions from this large background of possible matches is challenging.

IN VIVO FUNCTION OF miRNA GENES

The miRNA-processing factors Dicer, Dgcr8, Drosha and Ago2 are essential for viability in mice. Knockout mice individually lacking these key miRNA-processing genes died during early gestation with severe developmental defects (33–36). Therefore, to study the phenotypes of mature miRNA loss at later time points, Cre-inducible conditional knockout mice lacking Dicer and Dgcr8 have been generated (34,37–39). Although these knockout studies clearly demonstrated the importance of miRNAs on specific developmental stages and tissues, it is not clear which miRNAs are responsible for the observed phenotypes. Individual miRNA knockouts were first reported in 2007 by four independent groups (40–43). Following these initial reports, the number of miRNA knockouts published has greatly increased (Table 1). These knockouts largely emerged from cardiovascular and immune fields, presumably influenced by the focused interests of laboratories active in these areas.

ESSENTIAL ROLES OF miRNAs DURING EMBRYONIC DEVELOPMENT

A highly conserved muscle-specific miRNA, *miR-1*, is one of the first miRNAs ablated in mice (43,44). Deletion of *miR-1* in flies caused death during embryonic development or at larval stages with a spectrum of defects in muscle differentiation (45,46). Although mammalian *miR-1* is duplicated as *miR-1-1* and *miR-1-2*, targeted deletion of just *miR-1-2* in mice resulted in ~50% embryonic lethality with cardiac defects, implying the functional haploinsufficiency of *miR-1* (43). This result strongly suggests that *miR-1* is essential for cardiac development, though generation of a double knockout is necessary for further evaluation. Another example is the *miR-17–92* cluster. Genetic ablation of this cluster caused a perinatal death with lung and heart defects, along with abnormal B cell development in mice (47). In addition, transgenic mice with higher expression of *miR-17–92* in lymphocytes resulted in premature death with lymphoproliferative disease and autoimmunity (48). Combined loss- and gain-of-function

mice demonstrate that the *miR-17–92* cluster is absolutely required for the development and survival of mice (47,48).

Targeted deletion of *miR-126*, an endothelial cell-specific miRNA located in an intron of *EGFL7*, caused embryonic lethality in 50% of nulls with leaky vessels and hemorrhage (49,50). Surviving mutant mice displayed defects in postnatal retinal vascularization, indicating that *miR-126* plays key roles in vascular integrity and angiogenesis. The partial embryonic lethality observed in mice suggests the role of *miR-126* as a modulator of the angiogenesis program, rather than an on–off switch. It is worth mentioning that angiogenic defects of this locus (*EGFL7* and *miR-126*) were previously attributed to the deletion of *EGFL7* (51). By generating knockout mice lacking specifically either *miR-126* or *EGFL7*, Kuhnert *et al*. (49) clearly demonstrated that the vascular phenotypes were caused by deletion of *miR-126* and not by loss of *EGFL7*. Though this is the first reported case where an intronic miRNA confounded the analysis of a gene knockout in mouse, other knockout mice already made may potentially have similar complications (52).

REDUNDANCY AND/OR COOPERATION OF COEXPRESSED AND RELATED miRNAs

In mammals, many miRNAs exist as duplicates or highly similar genes, which raises the question of both functional redundancy and cooperation. An example of redundancy is demonstrated by *miR-133a-1* and *miR-133a-2*. Deletion of either *miR-133a-1* or *miR-133a-2* exhibits no obvious defects, whereas deletion of both results in late embryonic and neonatal death in approximately half of the mice with ventricular septal defects and cardiac chamber dilation (53).

The occurrence of miRNA clusters containing multiple, highly similar miRNAs is common in mammals. One of the best examples is the *miR-17–92* cluster. *The miR-17–92* cluster is often amplified and overexpressed in human cancers and has been shown to cooperate with *c-Myc* in a mouse model of B cell lymphoma (54,55). Though this cluster has been well demonstrated for its role in tumorigenesis, the *in vivo* functional studies are further complicated because of its paralogous clusters, the *miR-106a–363* and *miR-106b–25* clusters. All three clusters collectively comprise 15 miRNAs from 4 different families, and the genomic organization is evolutionary conserved in vertebrates. Ventura *et al*. (47) generated mice lacking these clusters, individually and in combination. Whereas mice lacking either the *miR-106a–363* or the *miR-106b–25* cluster were viable with no obvious abnormalities, mice lacking the *miR-17–92* cluster died shortly after the birth with heart and lung defects. Compounding double and triple knockouts including the *miR-17–92* cluster displayed more severe cardiac defects as well as additional defects not found in *miR-17–92* single knockouts, suggesting genetic interactions among the related miRNA clusters.

Another example of cooperation among related miRNAs can be found in three muscle-specific miRNAs, *mirR-208a*, *miR-208b* and *miR-499*. These miRNAs are located within introns of three corresponding myosin genes, *Myh6, Myh7* and *Myh7b*. The seed sequences of *miR-208a* and *miR-208b* are identical, and *miR-499* shares six of the seven seed nucleotides with *miR-208*. Additionally, the genomic organization of the myosin genes and their intronic miRNAs is well conserved from fish to human, suggesting critical roles of these miRNAs in regulating muscle contractility through the expression of the host myosin genes. Whereas mice lacking *miR-208a* were viable and had no obvious phenotype under normal conditions, deletion of *miR-208a* inhibited up-regulation of *Myh7* (the host gene for *miR-208b*) in response to pressure overload in the heart. In addition, loss of *miR-208a* also abrogated expression of *miR-499* in normal hearts (42,56). Conversely, transgenic mice over-expressing *miR-208a* displayed an up-regulation of *Myh7* expression, and transgenic *miR-499* mice were sufficient to functionally replace *miR-208a* in adult hearts. Combined results from these two reports demonstrate that these myosin-encoded miRNAs act within a network to control myosin expression and muscle performance (42,56). The regulation of host myosin gene expression by these intronic miRNAs is quite fascinating and this regulation is reminiscent of the relationship between Hox miRNAs such as *miR-10a* and *miR-196* and their neighboring *HOX* genes (57).

THE QUEST FOR miRNA TARGETS

Since miRNAs may direct post-transcriptional silencing by pairing to target mRNAs, identifying these target mRNAs has been a primary focus. Many computational target-prediction tools have been developed, which generally predict up to a couple of hundreds of targets per miRNA (58). To overcome limitations of computational prediction and validate the predicted targets, proteomics approaches and high-throughput sequencing assays have been developed (32,59–61). Still, identification of targets is a major challenge in the field.

It has been proposed that a single miRNA can repress hundreds of target transcripts within a cell to dampen protein output (62). This model is strongly supported by recent proteomics approaches showing widespread changes in protein synthesis, each to a modest level, by a single miRNA (59,63). This model of fine-tuning is further supported by evidence gathered from knockout mice lacking *miR-155*, *miR-143/145*, *miR-144/451* and *mir-15a/16-1* (40,41,64–68). In particular, large numbers of identified *miR-143* and *miR-145* targets function within the actin regulatory network and this is consistent with structural modifications found in the aorta of mice lacking these miRNAs (64,66). Another example is highlighted by studies of *miR-15a* and *miR-16-1* in regulating B cell proliferation, where multiple miRNA targets control cell-cycle progression (67).

There is also strong evidence that some miRNAs can control a specific gene regulation program through a few major targets, suggesting they may function as switches. An elegant example of a switch is represented by *miR-150* and *c-Myb*. Opposite expression pattern of *miR-150* and *c-Myb* during B cell differentiation and targeting of *c-Myb* by *miR-150 in vitro* have been well documented (69,70). In accordance with this, deletion of *miR-150* in mice caused increased *c-Myb* expression and B1 cell expansion (71).

Using an *miR-150* over-expression mouse, Xiao *et al*. (71) also demonstrated that increased levels of *miR-150* reduced *c-Myb* levels and B1 cell population in a dose-dependent manner. Combined results from both loss- and gain-of-function of *miR-150* clearly indicate that this miRNA is responsible for B1 cell differentiation *in vivo* through *c-Myb* regulation. This single miRNA–single target relationship for a particular biological process is well supported by miRNAs identified through genetic screens during *C. elegans* development such as *lin-4, let-7* and *lsy-6* (1–3,72).

Another example of single miRNA–single target is highlighted by *miR-223* and *Mef2c*. Genetic ablation of *miR-223*, a myeloid-specific miRNA, caused an expanded granulocyte compartment and increased circulating neutrophils in mice (73). The defects in granulocyte expansion were attributed to the increase of a transcription factor, *Mef2c*, known to modulate cell fate decisions between monocytes and granulocytes (74). To examine the functional importance of *Mef2c* up-regulation as part of the *miR-223* circuitry *in vivo*, Johnnidis *et al*. (73) generated myeloid-specific *Mef2c* null mice on the *miR-223* null background. Double-knockout mice displayed a corrected level of peripheral neutrophils, providing strong genetic evidence that *Mef2c* is a major target of *miR-223* during early myeloid progenitor differentiation. It should be noted that a recent proteomics approach demonstrated the de-repression of numerous proteins in neutrophils lacking *miR-223* (59). These seemingly conflicted observations bring an interesting point that, although a single miRNA can repress hundreds of genes, only a few of these may be critical for a particular phenotype.

The vast number of target genes presented by proteomics or expression analysis present the major challenge to decide which mRNAs are bona fide targets. An ideal approach is to disrupt only the interaction between single miRNA and single target *in vivo* and observe the phenotypic consequences. This was done by two independent groups for *miR-155* and *activation-induced cytidine diaminase* (*AID*) using either knockin or BAC trangenic approches (75,76). The role of miR-155 in germinal center B cell development was demonstrated previously (40,41). *AID* was identified as one of more than 60 genes up-regulated in *miR-155*-deficient B cells (77). To determine whether *miR-155* directly regulates *AID* expression, mice carrying a mutation within the putative *miR-155*-binding site in the 3′-UTR of *AID* were generated (75,76). These genetically modified mice had an increase in both *AID* mRNA and protein levels and furthermore displayed induced *Myc-Igh* translocation and impaired affinity maturation phenotypes found in *miR-155* knockout mice. Targeted mutagenesis of miRNA-binding sites within target mRNAs is ultimately required to claim the miRNA–target relationship.

ROLE OF miRNAs AS MODIFIERS OF PATHOGENESIS

A skeletal muscle-specific miRNA, *miR-206*, is the most up-regulated miRNA in the *G93A-SOD1* transgenic mouse, a mouse model of amyotrophic lateral sclerosis (ALS), (78). Whereas *miR-206* knockout mice do not have any obvious abnormalities, those with a low copy number of *G93A-SOD1* display accelerated disease progression and die prematurely, suggesting a compensatory role of *miR-206* in disease progression. Williams *et al*. (78) demonstrated further that *miR-206* plays a critical role in the formation of new neuromuscular junctions after nerve injury, in part by repressing histone deacetylase 4 (*HDAC4*). Consistent with this finding, deletion of *HDAC4* in skeletal muscles showed opposing effect of *miR-206* deletion on re-innervation after injuries. These results strongly implicate *miR-206* as a modifier of ALS pathogenesis.

The *miR-15a/16-1* cluster is located inside of a noncoding RNA, deleted in leukemia 2 gene (*DLEU2*), long suspected as a candidate gene for chronic lymphocytic leukemia (CLL) (79). Point mutations within the primary precursor were found in a subset of human CLL patients and a mouse model of CLL phenotypes (80,81). Genetic ablation of the *miR-15a/16-1* cluster displayed B cell-autonomous lymphoproliferative disorders (67). In addition, re-introduction of the *miR-15/16* cluster into a human patient cell line dramatically reduced proliferation. Together, these results indicate that the *miR-15a/16-1* cluster modulates CLL pathogenesis in mammals.

Genetic ablation of *miR-375*, a pancreatic islet-specific miRNA, resulted in chronic hyperglycemia with increased total α-cell numbers and moderately decreased β-cell mass (82,83). In contrast, pancreatic islets of leptin-deficient obese mice, a model of increased β-cell mass, had increased expression of *miR-375*. The role of *miR-375* in regulating β-cell mass was further demonstrated by deletion of this miRNA in the obese mice. The mice deficient in both *miR-375* and leptin showed dramatic β-cell loss and developed severe insulin-deficient diabetes, suggesting that *miR-375* is essential for mediating metabolic stress through β-cell compensation.

A cartilage-specific miRNA, *miR-140*, is reduced in patients with osteoarthritic cartilage (84,85). In order to examine the role of *miR-140* in cartilage development and homeostasis, Miyaski *et al*. (86) generated both knockouts lacking *miR-140* and transgenic mice over-expressing *miR-140* in a cartilage-specific manner. Whereas young knockouts showed only mild skeletal phenotypes with short stature, the knockout mice displayed accelerated onset of age-related osteoarthritis-like changes. Conversely, the transgenic mice were resistant to antigen-induced arthritis, suggesting a role for *miR-140* in cartilage homeostasis.

Vascular smooth muscle cell plasticity is a key for vascular pathogenesis such as arteriosclerosis. Two clustered miRNAs, *miR-143* and *miR-145*, are highly expressed in vascular smooth muscle cells, and genetic ablation of this cluster displays vascular smooth muscle cell defects (64–66). The most striking observation from *miR-143/145* double-knockout mice was reduced neointima formation following carotid ligation injury reported by Xin *et al*. (66). This finding is contradictory to the observation that administration of *miR-143* or *miR-145* immediately following balloon injury reduced neointima formation (64,87). Nonetheless, these observations support critical roles of *miR-143/145* in mediating vascular smooth muscle cell programs in response to injury. Disparities between genetic modification and transient *in vivo* studies are

not rare. In the case of *miR-144/451* studies, genetic deletion and lentiviral decoy approaches resulted in different outcomes (68,88). This issue brings forth concerns in the field regarding transient *in vivo* experiments, although these approaches are useful in dissecting molecular mechanisms.

Based on observations from miRNA knockout models and rapidly accumulating human miRNA-disease association data, it seems clear that many miRNAs can function as modifiers of pathogenesis in response to stress and injuries (9). These observations further highlight the promise of miRNAs as therapeutic targets. Depending on whether a disease-associated miRNA acts in a pathogenic or compensatory manner, miRNA inhibitors or mimics may be further developed as therapeutics. In addition, the discovery of miRNAs as modifiers of disease processes can help identify cellular effectors and define molecular mechanisms of disease processes.

CLOSING REMARKS

Observations from reviewed knockout mouse models indicate that miRNAs can control expression of numerous functionally related proteins to exert their biological functions. However, some miRNAs can exert profound physiological effects by targeting only a few genes. It appears that different miRNAs can exert their functions by either acting as fine tuners or switches. Modes of action may vary from one miRNA to anther, over the course of development, or in a tissue-dependent manner.

Reviewed knockout studies also indicate that genetic ablation of miRNAs may not result in obvious phenotypes (53,78,89). In worms, $<10\%$ of systematic miRNA deletions cause embryonic death or grossly abnormal phenotypes (90). No apparent phenotypes could be caused by redundant functions of other related miRNAs or complex regulatory network buffering (58). Here, the miRNA–target mRNA interactions presumably fall within complex regulatory networks with multiple pathways and feedback controls that enable maintenance of homeostasis despite a defective node in the network. The fact that many miRNA knockouts exhibited phenotypes only under stress overloads or injuries supports the notion of the regulatory network buffering. However, we expect that genetic ablation of miRNAs in mice would display more discernible phenotypes compared with deletions in worms, because there are many mouse disease models and advanced analytical tools available in mice as highlighted in some knockout models (42,91). Since miRNAs constitute one of the largest classes of gene regulatory elements in mammals, deciphering their mode of action and their physiological roles is absolutely essential to understand biological systems.

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