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# microRNAs as Developmental Regulators

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**SUMMARY**

The field of miRNA biology is relatively young, but its impact on our understanding of the regulation of a wide array of cell functions is far-reaching. The importance of miRNAs in development has become nearly ubiquitous, with miRNAs contributing to development of most cells and organs. Although miRNAs are clearly interwoven into known regulatory networks that control cell development, the specific modalities by which they intersect are often quite distinct and cleverly achieved. The frequently emerging theme of feed-back and feed-forward loops to either counterbalance or reinforce the gene programs that they influence is a common thread. Many of these examples of miRNAs as developmental regulators are presently found in organs with different miRNAs and targets, whereas novel, unexpected themes emerge in the context of mouse development as we learn more about this rapidly developing area of biology.

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## Outline



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## 1 INTRODUCTION

Development is controlled by specific factors that can direct cell fate, differentiation, or behavior by controlling entire networks of gene expression. Classically, these "factors" have fallen into the category of proteins known as transcription factors that directly or indirectly bind DNA elements at specific genomic loci and control the transcription of nearby genes. Transcription factors have several characteristics that make them ideal regulators of development. Perhaps the most important characteristic is the ability of a single transcription factor to control the expression of numerous genes to execute whole cellular or organ programs in a coordinated fashion. Transcription factors are themselves highly regulated at the level of their expression and activity through chemical modifications and interaction with transcriptional coactivators or corepressors, providing flexibility and context specificity to their functions.

Given the characteristics of developmental regulators, it is perhaps not surprising that small noncoding RNAs belonging to the microRNA (miRNA) family have also emerged as important regulators of development. miRNAs are a large class of evolutionarily conserved, small, noncoding RNAs, typically 20–26 nucleotides in length, that primarily function posttranscriptionally by interacting with the 3′ untranslated region (UTR) of specific target mRNAs in a sequence-specific manner. More than 500 miRNAs are encoded in the human genome, and each is thought to target 50 to >100 mRNAs (messenger RNAs), resulting in mRNA degradation or translational inhibition. Interactions between miRNAs and mRNAs are largely believed to require sequence homology in the 5′ end of the miRNA, but significant variance in the degree of complementation in the remaining sequence allows a single miRNA to target a wide range of mRNAs, often regulating multiple genes within a common pathway.

The first described animal miRNA, *lin-4*, was cloned in a forward genetic screen and characterized as a translational repressor of developmental timing in *Caenorhabditis elegans*, targeting the 3′ UTR of *lin-14* mRNA (Lee et al. 1993). However, owing to lack of homology of *lin-4* in other species, it was considered a genetic peculiarity specific to *C. elegans*. Years later, a second miRNA, *let-7*, was cloned. It targeted the 3′ UTR of the highly conserved mRNA *lin-41*, another heterochronic gene. However, *let-7* was highly conserved across species, providing the first indication that miRNAs might be widely used across species to titrate protein expression (Pasquinelli et al. 2000; Reinhart et al. 2000). Through small RNA cloning efforts, it soon became clear that miRNAs were widespread in the genomes of all eukaryotes (Lee and Ambros 2001). More than one-third of mRNAs in the mammalian genome are thought to be regulated by one or more miRNAs (Chaudhuri and Chatterjee 2007).

Despite advances in miRNA discovery, the role of miRNAs in physiologic and pathophysiologic processes is just emerging. It has become clear that miRNAs have diverse roles in fundamental biological processes such as cell proliferation, differentiation, apoptosis, stress response, and tumorigenesis—processes that are vitally important for development.

## 2 miRNA BIOGENESIS, ORGANIZATION, AND TARGET RECOGNITION

miRNAs regulate gene expression at the posttranscriptional level by mRNA deadenylation, translation repression, or miRNA-mediated mRNA decay (Fig. 1). Mature miRNAs are formed in a multistep biological process involving critical endonucleases. miRNAs are initially transcribed from the genome into long (several kilobases) 5′ capped polyadenylated (poly[A]) primary transcripts (pri-miRNAs) by RNA polymerase II (Pol II) (Cai et al. 2004). Some miRNAs interspersed among repetitive DNA elements such as Alu repeats (5′ AG/CT 3′) can also be transcribed by RNA polymerase III (Borchert et al. 2006). The miRNA-encoding portion of the pri-miRNA forms a hairpin structure that is recognized and cleaved in the nucleus by a microprocessor complex. This complex consists of the double-stranded RNA-specific nuclease Drosha and its cofactor DiGeorge syndrome critical region 8 (DGCR8) (Landthaler et al. 2004). The resulting ~70-nucleotide hairpin precursor miRNA (pre-miRNA) is exported to the cytoplasm by the RAN-GTP-dependent nuclear transport receptor exportin-5 that acts by recognizing a 2–3 base pair overhang of the pre-miRNA stem-loop structure (Bohnsack et al. 2004; Zeng and Cullen 2004). The pre-miRNA is further processed by a complex of the RNase III-like ribonuclease Dicer and the transactivator RNA-binding protein, which cleaves the pre-miRNA to release the mature miRNA duplex.

An asymmetry in the relative thermodynamic stability of the 5′ ends of the miRNA duplex results in preferential loading of the less stable ~22-nucleotide strand into the RNA-induced silencing complex (RISC); the other strand is degraded, although in some cases, both strands are incorporated into the RISC (Khvorova et al. 2003; Schwarz et al. 2003; Ro et al. 2007). The RISC helps to mediate miRNA:mRNA interactions and subsequent mRNA repression or destabilization (Gregory et al. 2005). miRNAs typically bind to the 3′ UTRs of their mRNA targets with imprecise complementarity. Typically, the degree of Watson–Crick base pairing between bases 2 and 7 (the "seed region") at the 5′ end of the miRNA is critical for binding mRNA targets

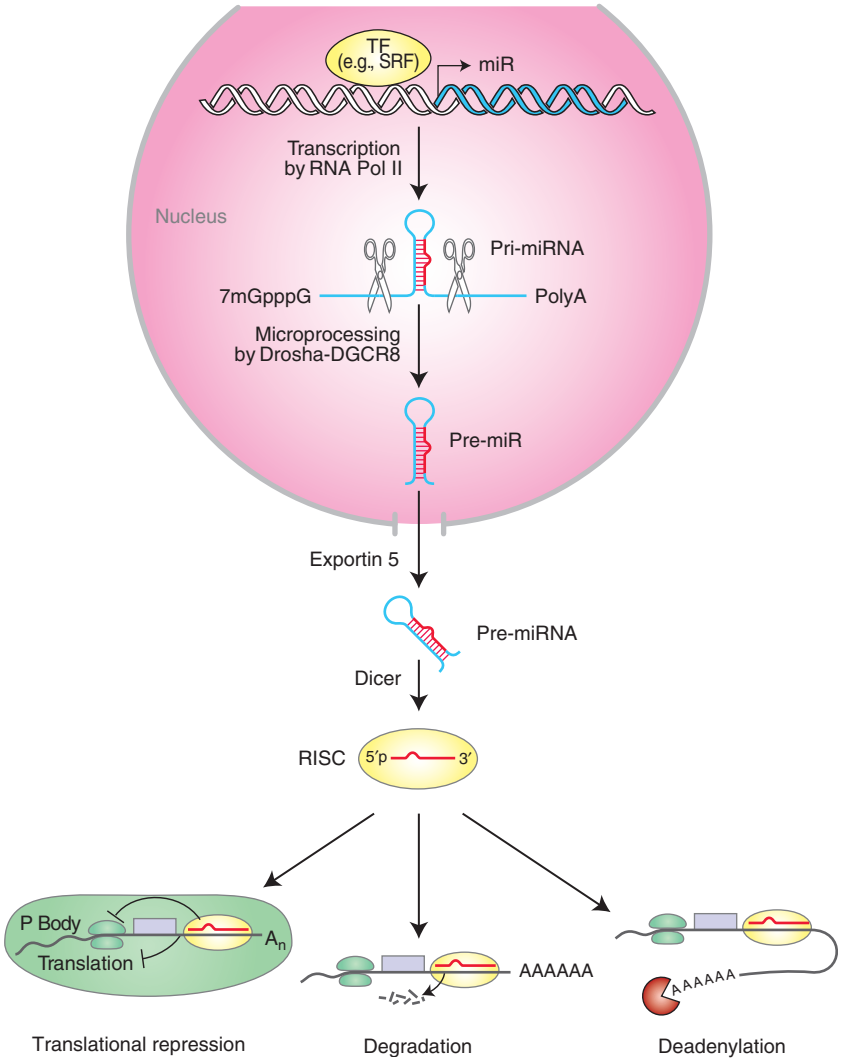


**Figure 1.** miRNA biogenesis. Schematic representation of miRNA biogenesis and function. Transcription of miRNA genes is typically mediated by RNA (Pol II). The initial miRNA-containing transcript, termed primary miRNAs (pri-miRNAs), can range from a few hundred nucleotides to several kilobases long. Inside the nucleus, the pri-miRNA has a characteristic stem-loop structure that can be recognized and cleaved by the ribonuclease III (RNase III) endonuclease Drosha along with its partner DGCR8 (DiGeorge syndrome critical region 8 gene; also known as *Pasha*). The cleavage product, a ~70-nucleotide stem-loop pre-miRNA, is exported from the nucleus by exportin 5. In the cytoplasm, another RNase III enzyme, Dicer, further cleaves the pre-miRNA into a double-stranded mature miRNA (~21 nucleotides) that is incorporated into the RNA-induced silencing complex (RISC), allowing preferential strand separation of the mature miRNA to repress mRNA translation or destabilize mRNA transcripts through cleavage or deadenylation. SRF, serum response factor; TF, transcription factor. (Adapted from Zhao and Srivastava 2007.)

(Stark et al. 2005; Rajewsky 2006) and causing repression. RISC-bound miRNAs may also be sequestered away from translational machinery in processing bodies (P bodies) that act by recruiting poly(A) nucleases to help modulate deadenylation of mRNA and thereby prevent translation (Liu et al. 2005; Kim et al. 2006; Ezzeddine et al. 2007).

miRNAs can be found in exons or introns of noncoding transcripts with independent enhancer regulation and in the introns and 3′ UTRs of protein coding transcripts. They can also overlap with either an exon or an intron, depending on the alternative splicing pattern. In flies and worms, some miRNAs in intronic regions bypass Drosha processing and enter the miRNA biogenesis pathway as pre-miRNAs (Ruby et al. 2007). In many cases, miRNAs are clustered near other miRNAs, suggesting they may be coregulated transcriptionally and share cooperative regulatory roles.

Among the hundreds of miRNAs identified thus far, only a limited number have been assigned target mRNAs.

Several algorithmic databases have been designed for miRNA target prediction that rely, for the most part, on the following criteria: (1) conservation across species, (2) complementarity of the 5′ miRNA "seed match" to the 3′ UTR (~7 nucleotides) (Lewis et al. 2005; Rajewsky 2006; Zhao and Srivastava 2007), (3) G:U wobbles in the seed (Brennecke et al. 2005), (4) thermodynamic context of target mRNA-binding sites (i.e., mRNA targets located in regions of high free energy and unstable secondary structure are favored) (Zhao and Srivastava 2007), and (5) multiple miRNA-binding sites in 3′ UTR (Doench and Sharp 2004). These computational programs are continuously updated to integrate these criteria with knowledge from newly validated miRNA:mRNA interactions. Until more miRNA targets are validated, the precise mechanism of what makes one predicted target mRNA-binding site more desirable than others remains to be determined.

## 3 miRNAs INTEGRATE WITH TRANSCRIPTIONAL AND SIGNALING NETWORKS DURING DEVELOPMENT

### 3.1 miRNAs Are Required for Differentiation

The highly interconnected activities of cell-cycle control and differentiation underlie myriad developmental processes. Consider one of the earliest developmental events—the switch from pluripotent to lineage-specified cells, which is marked by down-regulation of pluripotency markers, activation of lineage-specific gene expression, and decreased self-renewal potential. These dramatic changes are accompanied by the up-regulation of many miRNAs. The RNA-binding protein DGCR8 is specifically required for miRNA biogenesis and its deletion in pluripotent cells depletes most active miRNAs (Wang et al. 2007). When placed under conditions that normally promote differentiation, *DGCR8*-null embryonic stem (ES) cells fail to fully down-regulate pluripotency markers and display limited expression of lineage-specific genes. *DGCR8*-null ES cells also have altered cell-cycle properties, dividing more slowly than control cells under conditions that maintain pluripotency. These results reveal that miRNAs, in general, are critical for attainment of the features that distinguish differentiated cells from pluripotent cells and for the initial stages of organismal development. Specific studies have since shown mechanisms of cell-cycle regulation by individual microRNAs that are highly expressed in ES cells (Fig. 2A).

### 3.2 miRNAs Regulate Gastrulation

Some of the earliest developmental decisions in vertebrate embryos occur during gastrulation when the germ layers—ectoderm, endoderm, and mesoderm—are formed. The evolutionarily conserved *miR-430/427/302* family is exclusively expressed during this stage of development in zebrafish, *Xenopus*, and mammals, respectively. Studies in both human ES cells and *Xenopus* embryos revealed the importance of these miRNAs in promoting mesendoderm formation and suppressing the neuroectoderm lineage (Rosa et al. 2009). Interestingly, this occurs through species-specific targeting of components of the TGF-β signaling pathway. Specifically, in human ES cells, the TGF-β antagonists Lefty1 and Lefty2 are targeted by *miR-302*, whereas the agonist Nodal evades targeting. In contrast, zebrafish *miR-430* targets the orthologs of both Nodal and Lefty (Choi et al. 2007) whereas the *Xenopus miR-427* targets Lefty orthologs and a subset of Nodal-related genes (Rosa et al. 2009). Therefore, although nature has used similar miRNAs to regulate mesendoderm differentiation in distantly related animals, these early developmental decisions are achieved through targeting of unique messages from a common signaling pathway in different species (Fig. 2B).

### 3.3 miRNAs Regulate Neural Development

Although many miRNAs are ubiquitously expressed, expression of some individual miRNAs is predominantly limited to a single organ or tissue. Two such miRNAs are *miR-9* and *miR-124a*, which are highly expressed in neurons and astrocytes of the brain. Together, these miRNAs indirectly modulate the phosphorylation status of STAT3, an important intracellular signaling molecule mediating the inhibition of neuronal terminal differentiation (Krichevsky et al. 2006; Delaloy et al. 2010). Inhibition of *miR-9* increases STAT3 phosphorylation, resulting in reduced neuronal development, whereas overexpression of *miR-9* and *miR-124a* decreases STAT3 phosphorylation, limiting development of the astrocytic lineage. This is but one example of how multiple miRNAs can converge on a single pathway to promote a common developmental outcome (Fig. 2C).

As translational repressors, miRNAs often promote developmental progression by limiting the expression of genes that support the self-renewing progenitor state. *miR-9* offers an example of this type of activity in the brain, where its expression is specifically limited to neurogenic regions and is up-regulated as neural differentiation proceeds. In contrast, *TLX*, a highly conserved orphan nuclear receptor that is critical for neural stem cell self-renewal, is expressed in neurogenic regions but is down-regulated as neurons differentiate. The overlapping expression and known functions for *miR-9* and *TLX* made *TLX* an attractive candidate among the lists of bioinformatically predicted *miR-9* targets, and indeed, *miR-9* was shown to directly target *TLX*, decreasing levels of the protein (Delaloy et al. 2010).

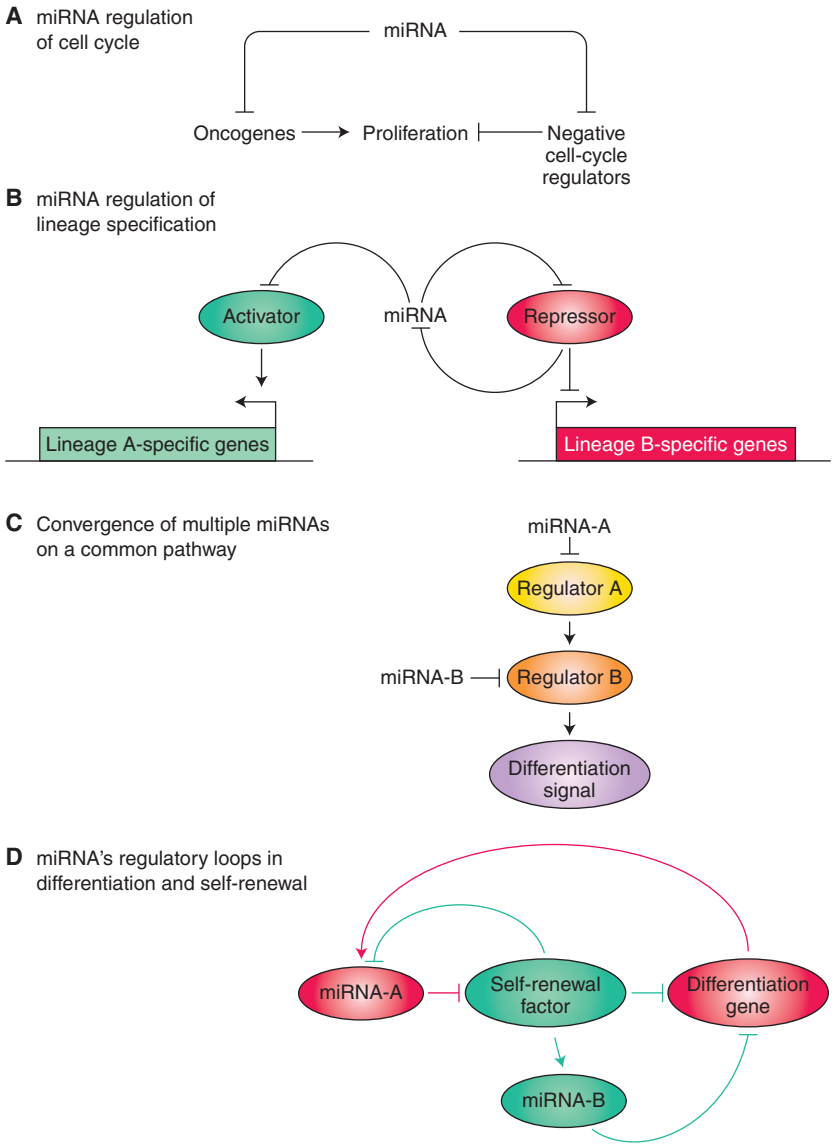


**Figure 2.** Common mechanisms of developmental regulation by miRNAs. (*A*) miRNAs modulate proliferation of differentiating cells by targeting either positive or negative cell-cycle regulators. (*B*) miRNAs act in regulatory loops to ensure complete commitment to specific cell lineages during development. (*C*) Multiple lineage-promoting miRNAs can converge on a single pathway to cooperatively regulate cell fate. (*D*) miRNAs act in regulatory loops with self-renewal genes to maintain the balance between progenitor cells and their differentiated progeny. (Adapted from Ivey and Srivastava 2010.)

More interestingly, *miR-9* and TLX were found to cooperate in an elaborate feedback loop (Zhao et al. 2009). TLX and the corepressor HDAC5 can bind the *miR-9* locus and repress its transcription, and *TLX*-null mice consequently express elevated levels of *miR-9*. Thus, TLX maintains its own protein levels by repressing its repressor, *miR-9*. Such feedback loops are commonly used during the switch from progenitor cell to differentiated cell and can help to maintain the delicate balance between proliferating progenitor cells and their differentiated progeny (Fig. 2D).

### 3.4 miRNAs Regulate Muscle Development

Among the first miRNAs to be identified as major regulators of lineage determination were those promoting the formation of muscle (Kwon et al. 2005; Zhao et al. 2005). Since then, miRNAs have been realized as being powerful regulators of cardiac, skeletal, and smooth muscle lineages, using clever regulatory mechanisms impinging on many previously described pathways. Two such miRNAs, *miR-1* and *miR-133*, are co-transcribed from a single locus and

are uniquely expressed in skeletal and cardiac muscle cells and their progenitors (Fig. 3) (Zhao et al. 2005; Chen et al. 2006). The influence of *miR-1* in promoting muscle identity is so strong that misexpression of this single miRNA in fibroblasts is sufficient to largely transform their gene program to more closely resemble muscle cells (Lim et al. 2005). Indeed, *miR-1* can promote the differentiation of skeletal muscle from myoblast precursors, in part by targeting a repressor of the muscle master regulator Mef2c that further drives expression of *miR-1* (Chen et al. 2006). Misexpression of *miR-1* in either mouse or human ES cells causes them to favor the muscle cell fate (Ivey et al. 2008). *miR-1* also provides an example of a tissue-specific regulator of cell cycle because its overexpression

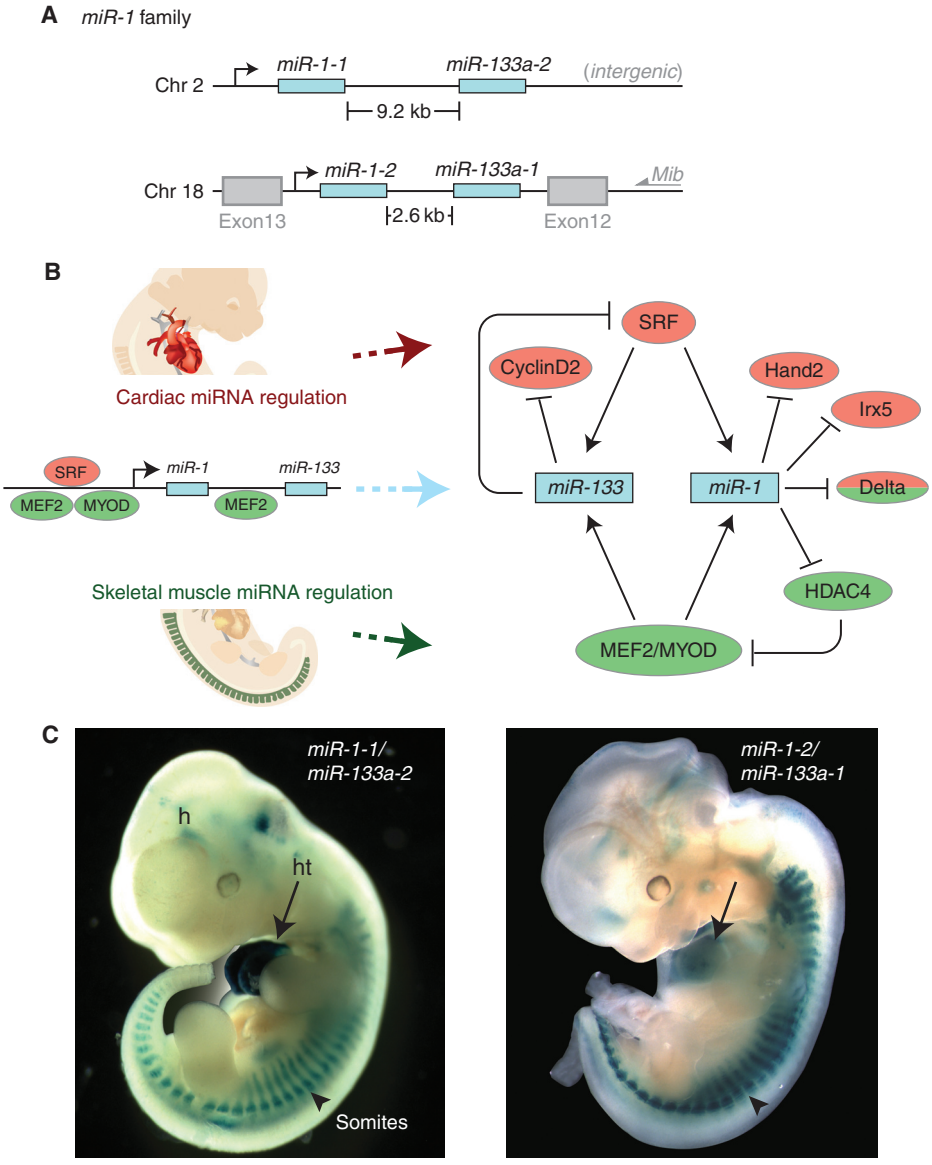


**Figure 3.** Summary of *miR-1* and *miR-133* genomic organization, regulation, and expression during cardiogenesis. (*A*) Chromosome locations of *miR-1* and *miR-133a* orthologs. The *miR-1-2*/*miR-133a-1* cluster is intragenic, and the *miR-1-1*/*miR-133a-2* cluster is intergenic. *miR-1*/*133a* clusters are transcribed as bicistronic transcripts. (*B*) Cardiac- (red) and muscle- (green) specific expression of *miR-1* and *miR-133* clusters is regulated by SRF and myogenic transcription factors Mef2 and MyoD. Targets of *miR-1* and *miR-133* that regulate cardiac or skeletal muscle are shown. (*C*) LacZ directed by an upstream enhancer of the *miR-1-2*/*miR-133a-2* cluster and *miR-1-1*/*miR-133a-1* cluster, respectively, shows expression in the heart (ht) and somites (arrowhead) at mouse embryonic day 11.5. (From Cordes and Srivastava 2009; adapted, with permission.)

in developing mouse heart muscle leads to premature cell-cycle exit (Zhao et al. 2005), whereas a decrease in *miR-1* in mice causes cardiac developmental defects and persistent postnatal cardiomyocyte karyokinesis (Zhao et al. 2007).

Interestingly, both *miR-1* and *miR-133* potently direct pluripotent cells to form mesoderm while actively suppressing alternative lineages (Ivey et al. 2008). The results of many studies indicate that *miR-133* acts in partial opposition to *miR-1*, promoting muscle progenitor expansion and preventing terminal differentiation (Chen et al. 2006; Ivey et al. 2008). This may occur, in part, through *miR-133* repression of cyclin D2 (Liu et al. 2008) as well as serum response factor (SRF) (Chen et al. 2006), which controls differentiation and proliferation of muscle cells through interaction with specific cofactors. SRF and Mef2 directly regulate transcription of *miR-1* and *miR-133* in the heart, whereas skeletal muscle expression is dependent on MyoD and Mef2. Thus, these two coexpressed miRNAs have perfected a careful balancing act, regulating cardiac and skeletal muscle cell proliferation and differentiation through the establishment of feed-forward and feed-back loops integrated into known muscle cell networks and cell-cycle regulatory pathways (Fig. 3).

*miR-1* and *miR-133* are among a cohort of numerous miRNAs whose transcription is directed by and dependent on the developmental regulator SRF (Niu et al. 2008). In the absence of SRF, mesoderm differentiation is weak and delayed. Surprisingly, development of mesoderm progenitors can be partially rescued by forced expression of either *miR-1* or *miR-133* in differentiating mouse ES cells, despite the fact that many genes are dysregulated in the *SRF*-null state (Ivey et al. 2008; Niu et al. 2008), further highlighting the vast potential of individual miRNAs in promoting development of specific cell types.

Another cotranscribed pair of miRNAs under control of SRF is *miR-143* and *miR-145*. These two miRNAs are critical regulators of smooth muscle cells that uniquely oscillate between proliferative or more quiescent, differentiated states. Cotranscribed *miR-143* and *miR-145* cooperatively target a network of transcription factors, including *Klf4* and *Elk-1*, to promote differentiation and repress proliferation of smooth muscle cells (Cordes et al. 2009). Given their intercalation into these major regulatory pathways, their ability to direct differentiation of multipotent progenitors was also investigated. Indeed, *miR-145* was able to potently and rapidly direct the differentiation of multipotent neural crest stem cells into smooth muscle. Although *miR-145* was not required for smooth muscle differentiation in vitro or in vivo (Cordes et al. 2009), loss of *miR-145* resulted in a more proliferative, less differentiated state of smooth muscle in vivo (Zhang 2009).

### 3.5 miRNAs Regulate Bone Formation

Bone morphogenetic proteins (BMPs) are master regulators of cartilage and bone lineages. Secreted BMPs bind receptors that activate SMAD transcription factors, which in turn regulate expression of target genes. Mesenchymal stem cells, in vivo and in vitro, have the capacity to develop into many different cells types, including muscle, bone, and fat, with BMPs powerfully promoting their differentiation into bone. This occurs, in large part, through the activation of the transcription factor Runx2. However, the presence of BMP2 in mesenchymal stem cell media also rapidly modulates the expression of many miRNAs, some of which have been implicated in bone formation (Li et al. 2008).

Osteoblast differentiation can be controlled by a single miRNA and its dysregulation was also directly linked to human disease (Zhao et al. 2009). *miR-2861* was cloned from primary mouse osteoblasts and its expression was found to be primarily limited to osteoblasts. It was induced in bone marrow stromal cells treated with BMP2, concomitant with the activation of *Runx2*. Interestingly, blocking *miR-2861* expression in BMP-induced cells attenuated the accumulation of Runx2 protein, but did not change *Runx2* mRNA levels. Calcium deposition, a hallmark of bone development, was also decreased when *miR-2861* activity was blocked. These findings implicate this individual miRNA to be a key controller of bone development.

### 3.6 miRNAs Regulate Hematopoiesis

Like many tissues, bone marrow expresses a unique repertoire of miRNAs. During hematopoiesis, as lymphocytes develop and pass through various progenitor stages, distinct temporal expression of particular miRNAs is observed (Garzon and Croce 2008). These miRNAs can modulate the cell's response to its environment, thereby gently influencing the differentiation status during passage from a multipotent progenitor through progressively committed states. Some miRNAs enhance the self-renewing capacity of the cells in which they function, whereas others promote the progression to a more differentiated state. Although this theme is used in many types of developing cells in the human body, it has been most well characterized in the setting of hematopoiesis, owing to the detailed understanding and control of discrete developmental steps in this system.

A very early study implicating miRNAs in hematopoiesis identified three miRNAs, *miR-223*, *miR-142*, and *miR-181* as being primarily restricted to hematopoietic tissues of the mouse (Chen et al. 2004). In particular, *miR-181* was highly expressed in the thymus and predominantly found in the B-cell population. When hematopoietic progenitor cells ectopically expressing *miR-181* were transplanted into

lethally irradiated mice, the cells tended to favor the B-cell lineage over the T-cell lineage, providing an example of how an miRNA can promote development of a specific hematopoietic cell type.

## 4 APPLICATIONS AND FUTURE CHALLENGES

Many cancers are marked by the co-expression of genes associated with proliferation and differentiation, which does not occur to the same extent in normal tissues. In this way, cancer may be considered to represent a condition in which development has gone awry. For example, rhabdomyosarcomas, which are thought to arise from skeletal muscle progenitors, co-express markers of proliferation and myogenic differentiation. These cells are essentially poised to differentiate into muscle, but continue to self-renew. Interestingly, a wide array of rhabdomyosarcomas of varying origin and severity all show high levels of Met, which is associated with tumor growth and metastasis and is also a potential target of *miR-1*. Given that *miR-1* fails to be induced in cultured rhabdomyosarcoma cells, the effects of forced expression of *miR-1*, or its close relative *miR-206*, in the setting of rhabdomyosarcoma were investigated (Taulli et al. 2009; Yan et al. 2009). Introduction of *miR-1* or *miR-206* reduced proliferation and migration of rhabdomyosarcoma cells and promoted their differentiation and induced altered expression of more than 700 genes. Perhaps most important for the purposes of cancer therapy, *miR-1* repressed translation of Met and blocked the growth of rhabdomyosarcoma xenografts in vivo by promoting myogenic differentiation. These findings highlight the potential utility of miRNA modulation for cancer treatment owing to their combined influence on cell-cycle and differentiation-promoting effects.

miRNAs are clearly important for skeletal muscle development in a way that may also have economic importance. A study of Belgian Texel sheep, a breed coveted for their pronounced skeletal muscle hypertrophy, revealed a point mutation implicating miRNAs in lineage determination and phenotypic variation (Clop et al. 2006). Fine sequence mapping uncovered a single nucleotide A-to-G polymorphism in the 3′ UTR of the *GDF8* gene that is associated with this phenotype. This base change creates a novel *miR-1* binding site in the *GDF8* transcript, which encodes myostatin, a member of the TGF-β superfamily that is known to negatively regulate muscle mass. *miR-1* targeting causes a down-regulation of myostatin in the skeletal muscle of these sheep, resulting in increased skeletal muscle mass.

There is also evidence for miRNA dysregulation in human diseases including osteoporosis, a disease affecting the developmental potential of bone progenitors. Loss-of-function approaches in mice revealed that reduced activity of *miR-2861* causes a significant reduction in bone mass and osteoblast activity, suggesting that alterations of this evolutionarily conserved miRNA might lead to osteoporosis in humans. Indeed, among a cohort of 11 patients with primary osteoporosis, two siblings, each of whom lacked detectable *miR-2861*, were found to have a mutation in their *miR-2861* gene. This mutation resulted in a C-G base change in the stem of *pre-miR-2861* and was sufficient to block the biogenesis of mature *miR-2861* in vitro (Li et al. 2009). This is likely to be one of many future examples in which relatively modest changes in critical, fate-regulating miRNAs alter development, resulting in disease. Therefore, future genetic analysis of disease will need to take into account the potential involvement of miRNAs.

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