Cell Stem Cell
Minireview

MicroRNAs as Regulators of Differentiation and Cell Fate Decisions

Kathryn N. Ivey[1] and Deepak Srivastava[1,*]
[1]Gladstone Institute of Cardiovascular Disease and Departments of Pediatrics, Biochemistry and Biophysics, University of California San Francisco, 1650 Owens Street, San Francisco, CA 94158, USA
*Correspondence: dsrivastava@gladstone.ucsf.edu
DOI 10.1016/j.stem.2010.06.012

Unique expression domains, targets, and gain- and loss-of-function phenotypes of particular microRNAs have important implications for directed differentiation of stem cell populations and suppression of undesired cell types. We discuss this emerging topic, in part using muscle differentiation as a paradigm, and highlight common themes and unique modalities by which microRNAs exert their lineage-promoting or differentiation effects on multiple tissues.

Well before the identification and isolation of stem cells, developmental biologists pondered the phenomenon of lineage determination. What controls the fate of a cell and its progeny? Is it a mutable or fixed choice? And how do deviations in appropriate cell fate decisions result in birth defects and disease? In answering these questions during embryogenesis, investigators identified individual factors that had overwhelming influence on cell fate decisions, functioning as major switches by regulating gene expression. Although these findings helped answer basic questions about lineage determination, such master regulators and factors that influence their activity have also been employed to direct differentiation of stem cells into specific lineages, an important endeavor in today's efforts to develop regenerative therapies from the promise of stem cells.

Most major regulators of gene programs fall into the class of proteins known as transcription factors, which 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 cell fate. Perhaps the most important is the ability of a single transcription factor to control the expression of numerous genes to execute whole cellular or organ programs. Transcription factors are themselves highly regulated at the level of their expression and activity through chemical modifications and interaction with transcriptional coactivators or -repressors, providing flexibility and context specificity to their functions.

Given the characteristics of factors that regulate whole cellular programs, it is perhaps not surprising that small noncoding RNAs belonging to the microRNA (miRNA) family have emerged as a new class of cell lineage determinants. Over 650 miRNAs are known in humans and, like transcription factors, a single miRNA can regulate the expression of numerous genes. This effect generally occurs through direct Watson-Crick base-pairing of a small ~22 nucleotide mature miRNA to the 3′ UTR of partially complimentary messenger RNAs, largely involving the 5′ region of the miRNA known as the seed sequence. Interaction of the miRNA with cognate mRNAs typically results in either destabilization or suppressed translation of the mRNA targets. Like transcription factors, the spatial and temporal expression of miRNAs is highly regulated and responsive to changes in cellular status. Other aspects of miRNA regulation, including interactions with and chemical modification by various factors, are only recently being elucidated, adding to our understanding of the potential of these small molecules to promote programs that define the fate and character of developing cells.

Recent discoveries have revealed a model in which complex miRNA regulatory events are woven into the known transcription factor and signaling networks that control cell fate and differentiation, modulating their activity through positive and negative feedback loops to reinforce cellular decisions. Here, we provide a perspective on this topic, using muscle differentiation as a paradigm, and cite several other examples of miRNA regulators of lineage determination or differentiation. Although a complete literature survey is beyond this Minireview's scope, we have highlighted common themes and unique modalities by which miRNAs exert their lineage-promoting effects.

miRNAs Can Promote Cell Cycle Exit and Differentiation

miRNAs Are Required for Proper ESC Differentiation

The switch from pluripotent to lineage-specified cells is marked by downregulation of pluripotency markers, activation of lineage-specific gene expression, and decreased self-renewal. These dramatic changes are accompanied by the upregulation of many miRNAs. The RNA binding protein DGCR8 is specifically required for miRNA biogenesis and its deletion in embryonic stem cells (ESCs) depletes most active miRNAs (Wang et al., 2007). When placed under conditions that normally promote differentiation, *DGCR8* null ESCs fail to fully downregulate pluripotency markers and display limited expression of lineage-specific genes. *DGCR8* null ESCs also have altered cell cycle properties, which include 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 pluripotent and differentiated cells. Whereas some miRNAs, such as miR-145, function in promoting exit from the pluripotent state by targeting pluripotency factors (e.g., Klf4, Sox2, and Oct4), others stabilize the pluripotent state and are explored in the accompanying Minireview in this issue of *Cell Stem Cell* (Martinez and Gregory, 2010).

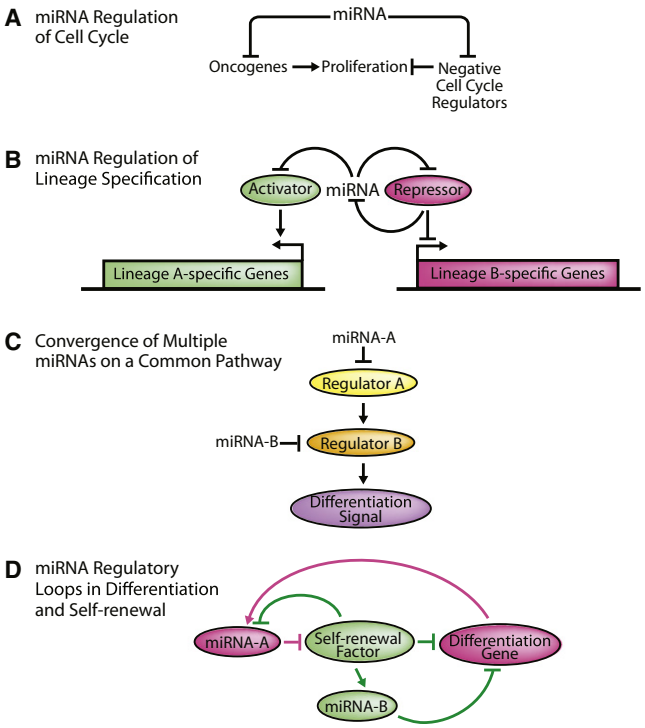

Figure 1. Common Mechanisms of Cell Fate Regulation by miRNAs
(A) miRNAs modulate proliferation of differentiating cells by targeting either oncogenes or negative cell cycle regulators.
(B) miRNAs act in regulatory loops to ensure complete commitment to specific cell lineages during differentiation.
(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.

miRNAs Target Cell Cycle Regulators

Differentiation is tightly linked with exit from the cell cycle. A common theme in the field of miRNAs is their targeting of cell cycle regulators in a variety of contexts (reviewed in Chivukula and Mendell, 2008). In particular, let-7, a family of closely related miRNAs, and the miR-15a/16-1 cluster have been repeatedly implicated as important regulators of the cell cycle and as potential human tumor suppressors. Numerous genes whose products promote the G_1/S or G_2/M transitions, including CDK6, CDC25A, and CCND2, are direct let-7 targets. In addition, let-7 indirectly influences cell cycle by negatively regulating oncogenes such as NRAS, KRAS, HMGA2, and c-MYC, which would otherwise promote proliferation. Similarly, the miR-15a/16-1 cluster downregulates expression of CDK6, CARD10, and CDC27, preventing cells from entering S phase.

Although let-7 and miR-15a/16-1 are generally known as cell cycle regulating miRNAs, many additional miRNAs include cell cycle regulators in their vast target repertoire. Conversely, miRNA function can itself be modulated by cell cycle state. For instance, relative levels of miR-29a and miR-29b, which are encoded by a single primary transcript, are regulated by selective miR-29b degradation in all cell cycle phases except for mitosis (Hwang et al., 2007). A hexanucleotide element found in miR-29b, but absent from miR-29a, directs import of miR-29b to the nucleus, where it is stabilized during mitosis, although the mechanism underlying increased stability during this specific phase of the cell cycle is still unknown as are the ultimate effects of this differential stability and unique localization.

In other instances, the specific outcome of a single miRNA-target interaction may be regulated by the cell cycle with rare examples of miRNAs activating translation in quiescent cells, whereas the same miRNA represses translation during proliferation (Vasudevan and Steitz, 2007). Although the degree to which individual miRNAs promote translational activation remains unclear, miRNAs do play an important role in controlling proliferation (Figure 1A) and are also poised to respond to changes in cell cycle.

miRNAs Promote Differentiation by Inactivating Transcriptional Repressors

Active transcriptional repression of lineage-specific genes reinforces the undifferentiated state. This stabilization is, in part, achieved by polycomb group proteins (PcGs), which occupy and repress promoters of specific developmental genes. Differentiation is associated with relieved repression of such fate-determining genes, but the mechanism for this switch in various lineages is poorly understood.

One of these PcG proteins, Ezh2, is expressed in pluripotent cells, but its protein levels are downregulated during skeletal muscle differentiation disproportionately compared to its mRNA levels. This observation suggests an active blockade of Ezh2 translation in this cell type upon differentiation. Indeed, miR-214 is activated during skeletal muscle differentiation and functions in a regulatory loop with Ezh2 (Juan et al., 2009). Specifically, Ezh2 represses miR-214 expression, but the downregulation of Ezh2 that coincides with the onset of skeletal muscle differentiation relieves this repression. miR-214 levels therefore increase, and miR-214 directly targets the Ezh2 3′ UTR to repress its translation. This interaction sets into motion a cascade of events that ensures complete differentiation of skeletal muscle cells. Although this example may be specific to skeletal muscle, similar reinforcing feedback loops involving tissue-specific miRNAs and PcG proteins are likely to exist (Figure 1B).

miRNAs Integrate with Transcriptional and Signaling Networks to Control Cell Fate and Differentiation

Species-Specific Regulation of TGF-β Signaling during Gastrulation

Some of the earliest cell fate decisions in vertebrate embryos occur during gastrulation when the germ layers—ectoderm, endoderm and mesoderm—are distinguished. 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 ESCs and *Xenopus* embryos revealed the importance of these miRNAs in promoting mesendoderm formation and suppressing the neuroectoderm lineage (Rosa et al., 2009). Interestingly, this regulation occurs through species-specific targeting of components of the TGF-β signaling pathway. Specifically, in human ESCs, 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 the Lefty

orthologs and a subset of the Nodal-related genes (Rosa et al., 2009). Therefore, although nature has employed similar miRNAs to regulate mesendoderm differentiation in distantly related animals, the cell fate decisions are achieved through targeting of unique messages from a common signaling pathway in different species.

miRNAs Regulate Neurogenesis

Expression of many miRNAs is predominantly limited to a single organ or tissue. Two such miRNAs are miR-9 and miR-124a. These miRNAs are coinduced during differentiation of neural progenitors into neurons and astrocytes in vitro and are highly expressed in the brain. Their temporal induction and unique expression pattern led to the hypothesis that they may influence neural differentiation from ESCs. Indeed, gain- and loss-of-function approaches demonstrated a role for miR-9 and miR-124a in formation and proliferation of the neural lineage from ESCs (Delaloy et al., 2010; Krichevsky et al., 2006). Together, these miRNAs modulate the phosphorylation status of STAT3, an important intracellular signaling molecule mediating the inhibition of neuronal terminal differentiation. Inhibition of miR-9 increases STAT3 phosphorylation, resulting in reduced neuronal differentiation, whereas overexpression of miR-9 and miR-124a decreases STAT3 phosphorylation, limiting astrocytic lineage differentiation. Although direct targets of the miRNAs that lead to altered STAT3 modification were not identified, this relationship is an example of how multiple miRNAs can converge on a single pathway to promote a common outcome (Figure 1C).

As repressors, miRNAs often promote differentiation 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, in which its expression is specifically limited to neurogenic regions and is upregulated 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 downregulated 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, thereby decreasing levels of the protein (Zhao et al., 2009).

More interestingly, miR-9 and TLX were found to exist 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 employed during embryonic development and can help to maintain the delicate balance between proliferating progenitor cells and their differentiated progeny (Figure 1D). Exploiting such regulation may also prove useful for expanding progenitor cells in vitro and differentiating them in a controlled manner.

miRNAs Regulate Muscle Differentiation

Among the first miRNAs to be identified as major regulators of lineage determination were those promoting the formation of muscle (Zhao et al., 2005). Since then, miRNAs have been realized as powerful regulators of cardiac, skeletal, and smooth muscle lineages employing clever regulatory mechanisms impinging on many previously described transcriptional pathways. Two such miRNAs, miR-1 and miR-133, are cotranscribed from a single locus and are uniquely expressed in skeletal and cardiac muscle cells and their progenitors (Chen et al., 2006; Zhao et al., 2005). The influence of miR-1 in promoting the muscle identity is so strong that mis-expression of this single miRNA in fibroblasts is sufficient to largely transform their gene program to that of 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, which further drives expression of miR-1 (Chen et al., 2006). Mis-expression of miR-1 in either mouse or human ESCs 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 given that its overexpression 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 post-natal 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 effect 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 upon MyoD and Mef2. Despite expression of miR-133 in skeletal and cardiac muscle, miR-133 represses skeletal muscle gene expression in the heart, suggesting context-specific target selection in individual tissues (Liu et al., 2008). 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 feedback loops integrated into known muscle cell networks and cell-cycle regulatory pathways.

miR-1 and miR-133 are among a cohort of numerous miRNAs whose transcription is directed by and dependent on SRF (Niu et al., 2008). In the absence of SRF, differentiation of mouse ESCs into mesoderm is weak and delayed. Surprisingly, progression of mesoderm progenitors can be partially rescued by forced expression of either miR-1 or miR-133 in differentiating mouse ESCs, in spite of the many genes that 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 specific cell fates.

Another cotranscribed pair of miRNAs under control of SRF is miR-143 and miR-145. These two miRNAs have recently surfaced as critical regulators of smooth muscle cells, which uniquely oscillate between proliferative or more quiescent, differentiated states. The 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 in vitro (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, which has the unique capacity to induce expression and synergize with the smooth muscle master regulator, Myocardin, in addition to targeting many other genes, 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 vivo or in vitro, loss of miR-145 resulted in a more proliferative, less differentiated state of smooth muscle in vivo (reviewed in Zhang, 2009).

miRNAs in Cell Fate Determination and Disease

In Hematopoiesis and Leukemia

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 (reviewed in 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 utilized in many types of differentiating cells in the human body, it has been most well characterized in the setting of hematopoiesis because of the detailed understanding and control of discrete differentiation 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 hematopoeitic 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 hematopoeitic 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 a miRNA can promote differentiation to a specific hematopoeitic fate.

Given the precise and dynamic miRNA expression seen during the process of hematopoiesis and the general control that these regulators impose on cell cycle and oncogene activity, it is perhaps not surprising that extensive miRNA dysregulation has been observed in leukemias and lymphomas (reviewed in Garzon and Croce, 2008). Among the changes that have been described are downregulation of the cell cycle miRNAs miR-15a and miR-16 and an upregulation of the oncogenic miR-17∼92 cluster. These differences will serve as diagnostic markers of disease and its severity, but may also provide targets for therapeutic intervention as well as for controlled progenitor cell expansion in vitro.

In Muscle Differentiation and Rhabdomyosarcoma

Many cancers are marked by the coexpression of genes associated with proliferation and differentiation, which does not occur to the same extent in normal tissues. For example, rhabdomyosarcomas, which are thought to arise from skeletal muscle progenitors, coexpress 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 because of their combined influence on cell cycle and differentiation promoting effects.

The potential consequences of polymorphisms in miRNA binding sites were exemplified by the discovery of a 3′ UTR variant that affected skeletal muscle cell fate in an inbred animal strain. A study of Belgian Texel sheep, a breed coveted for their pronounced skeletal muscle hypertrophy, revealed a single-nucleotide A-to-G polymorphism in the 3′ UTR of the *GDF8* gene that is associated with this phenotype (Clop et al., 2006). 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 negatively regulates muscle mass. miR-1 targeting causes a downregulation of Myostatin in the skeletal muscle of Texel sheep, resulting in increased skeletal muscle mass.

In Bone Formation and Osteoporosis

Bone morphogenetic proteins (BMPs) are master regulators of the 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 differentiate into many different cells types, including muscle, bone, or fat with BMPs powerfully promoting their differentiation into bone. This impact on bone formation 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).

Recently, a novel miRNA, miR-2861, that promotes osteoblast differentiation was discovered, and its dysregulation was directly linked to human disease (Li et al., 2009). miR-2861 was cloned from primary mouse osteoblasts and its human ortholog was identified. Upon further examination, expression of miR-2861 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 differentiation, was also decreased when miR-2861 activity was blocked. The findings suggest that miR-2861 promotes osteoblast differentiation.

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

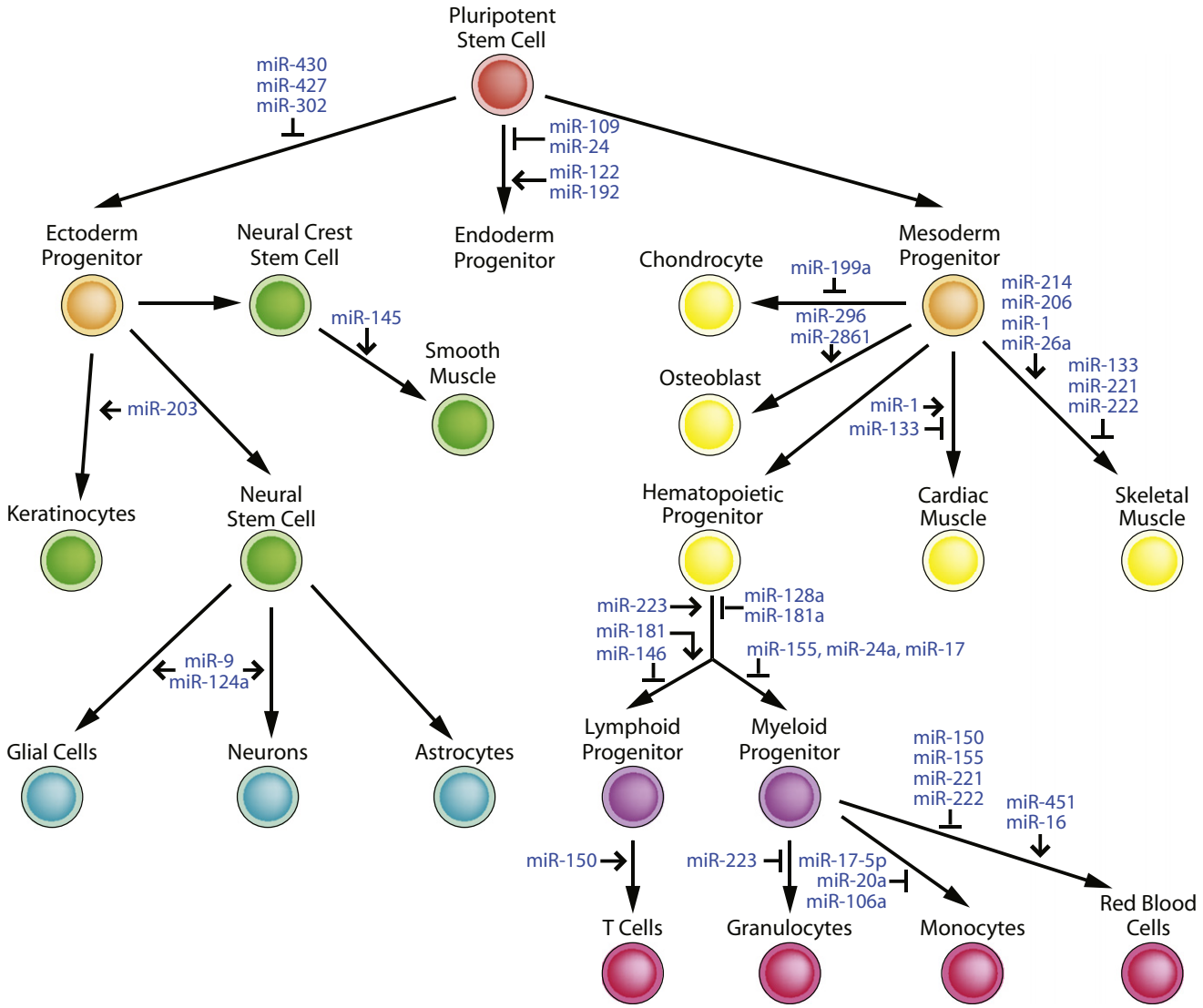

Figure 2. miRNAs Can Regulate the Specification or Differentiation of Numerous Cell Types
Schematic diagram showing progressive commitment and subsequent differentiation of various lineages from pluripotent stem cells. Some examples of the influence by miRNAs on specific cell fates are shown.

osteoporosis, two siblings, who lacked detectable miR-2861, were found to have a mutation in their *miR-2861* gene. This mutation resulted in a C-to-G base change in the stem of pre-miR-2861 and was sufficient to block the biogenesis of mature miR-2861 in vitro. This mutation was not identified in healthy controls and, interestingly, serum markers of osteoblast activity were lower in these individuals than in control patients, which is not necessarily the case in all instances of primary osteoporosis. This observation is likely to be one of many future examples in which relatively modest changes in critical, fate-regulating miRNAs alter cellular differentiation, resulting in disease.

Conclusions

Although the field of miRNA biology is relatively young, its impact on our understanding of the regulation of a wide array of cell functions is far-reaching. In particular, the importance of miRNAs in cell fate determination and differentiation, though initially surprising, has become nearly ubiquitous, with miRNAs contributing to the specification or differentiation of many cell types (Figure 2). Although miRNAs are clearly interwoven into known regulatory networks that control cell fate and differentiation, the specific modalities by which they intersect are often quite distinct and cleverly achieved. The theme of feedback and feed-forward loops to either counterbalance or reinforce the gene programs that miRNAs influence is a common emerging thread. As obstacles to efficient mRNA target identification are removed, the full array of miRNA-mediated gene regulation will be realized. It is likely that many of the initial examples of miRNAs as regulators of specific lineages will resurface in alternative settings with different miRNAs and targets, and novel, unexpected themes will almost certainly emerge as we learn more about this rapidly developing area of biology.

ACKNOWLEDGMENTS

We wish to acknowledge and apologize to the many contributors to this important field who we were not able to cite here due to formatting constraints and strict limits on references. D.S. is supported by grants from the NHLBI/NIH and the California Institute for Regenerative Medicine. D.S. is a member of the scientific advisory board of iPierian, Inc., and RegeneRx, Inc.

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