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Small RNAs have a big impact on regeneration

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Abstract

A number of lower vertebrates including urodele amphibians and teleost fish are remarkably adept at repairing and regenerating damaged tissues and organs. Freshwater planarians are even more amazing, capable of regenerating entire body plans from small amputated fragments. In contrast, mammalian regenerative capacity is quite limited but of intense interest, especially related to human health and disease. For those organisms capable of robust regeneration, a common theme is the use of stem cells to replace complex tissues. Key questions remain as to the origin of these cells, whether there are pools of such cells that migrate to injured regions or whether they are generated on site. Beyond their origin, how are the genetic pathways that enable differentiation into multiple cell types and tissues regulated? microRNAs (miRNAs) are small noncoding RNAs that have recently been shown to play important roles in controlling stem cell self-renewal, proliferation and differentiation. Some of these are thought to be required to maintain "stemness". Here, we summarize recent work on the role of miRNAs in stem cells and their roles during regeneration.

Keywords

regeneration; microRNAs; stem cells; zebrafish

Small RNAs in Regeneration

miRNA biogenesis

Recently, a new class of regulatory molecules, small noncoding RNAs referred to as microRNAs (miR-NAs), has been implicated in regeneration. miRNAs have been shown to regulate many cellular functions including cell specification, differentiation, proliferation and metabolism.[1–11] A key to understanding their role is to determine how many are encoded in animal genomes and what genes they regulate. miRNA biogenesis begins with primary transcripts synthesized by RNA polymerase II that form extended hairpin structures containing one or more miRNAs. Some of these primary transcripts originate from independent transcripts whereas others are encoded within the introns of mRNA transcripts. Primary transcripts are recognized in the nucleus by an RNase III enzyme, Drosha, which cleaves hairpin precursors for subsequent export to the cytoplasm in a Ran-GTP dependent process.[12,13] In the cytoplasm, hairpin precursors are recognized by another RNase III

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enzyme, Dicer, that produces RNA duplexes (~22 nucleotide) that are incorporated into RNA Induced Silencing Complexes (RISCs).[14–16] Either coincident or subsequent to RISC assembly, miRNAs are guided to their target mRNAs where they most often pair with 3′UTR sequences.[17,18] Partial complementarity leads to translational repression whereas perfect complementarity leads to cleavage of the target mRNA (Fig. 1). This process is highly conserved throughout development.

A number of studies have examined the expression patterns of miRNAs during development, differentiation and regeneration. A common theme is that more and more miRNAs are expressed as differentiation proceeds.[19,20] A smaller number are expressed in stem cells and at early stages of development, including some that are thought to be required to maintain "stemness".[21–25] For animals that are adept at repairing or regenerating damaged tissues, the role of stem cells during regeneration can be simplistically boiled down to two nonexclusive mechanisms. First, a reserve of stem cells are somehow recruited to the site of damage and differentiate to restore complex tissues and organs. Second, local stem-cell like cells near the site of damage de-differentiate to restore and repair the damage. Both scenarios propose the existence of some kind of stem cell which responds to signals that drive regeneration. Here, we will focus on recent work illustrating the role that miRNAs play in stem cell maintenance and proliferation followed by a review of recent work on the role of miRNAs during regeneration.

miRNAs in Stem Cells Maintenance

One intriguing possibility for how or why miRNAs may play a key role in regeneration is related to the finding that only a subset of miRNAs are expressed in stem cells.[8,26–28] Dicer deficient mice lack almost all pluripotent stem cells and die around embryonic day 7.5.[29] Similarly, mouse Dicer hypomorphs die mid-gastrulation[30] and in cultured cells, loss of Dicer inhibits embryonic stem cell proliferation.[31] Deletion of a DGCR8, a protein involved in nuclear processing of miRNA primary transcripts also leads to slowly proliferating ES cells that accumulate in G_1.[24] This is consistent with the fact that an intact miRNA biogenesis pathway is required for development and raises the possibility that early steps in embryonic development require the action of specific miRNAs to properly regulate stem cells. In line with this, restoration of multiple members of the *miR-290* family could rescue proliferation in DGCR8 deficient ES cells although they could not restore differentiation defects.[32] These miRNAs are referred to as ESCC miRNAs (ES cell-specific Cell Cycle regulating miR-NAs). Interestingly, proliferation and self-renewal can be blocked in ES cells by expression of *let-7* miRNAs indicating that distinct subsets of miRNAs regulate stem cell maintenance, proliferation and differentiation.[22] Broadly, this is consistent with findings that overall miRNA complexity increases with development. More and more miRNAs are expressed as cells differentiate into tissues and organs.[19,20,33,34] For *let-7*, this is additionally interesting because its expression is regulated by control of miRNA processing and maturation.[35–39] Mature *let-7* is readily detected in more differentiated cell types whereas the precursor accumulates in ES cells and de-differentiating tumor cells.[40]

One possibility for the above results is that *let-7* functions as an anti-stemness factor.[22] Since regeneration relies on recruitment of stem cells or de-differentiation of cells into stem-

cell like cells, it is logical to expect that *let-7* or similar miRNAs will need to be turned off during regeneration whereas opposing sets of miRNAs will need to be turned on. Two examples are *miR-196,* whose expression is elevated during early salamander tail regeneration.[41] In contrast, *miR-203* must be turned off to initiate fin regeneration and allow Lef1 expression.[23] For *miR-203*, the Fuchs and Candi groups showed that it represses stem cell activity in mice by downregulating *p63*, thereby repressing 'stemness'.[21,25] Further identification and understanding of the role that miRNAs play in ES self-renewal and proliferation is likely to shed light on similar involvement in regeneration and vice versa (Fig. 3).

miRNAs in Planaria

Planarians are small flatworms often found in freshwater and marine environments that are capable of remarkable feats of regeneration. Numerous stem-cell like cells, called neoblasts, make up nearly 30% of the total cells in planarians and their regenerative capacity allows re-creation of whole animals, even when cut into numerous pieces.[42–44] Using microarrays and in situ localization, a unique set of ~50–60 miRNAs were found to be highly expressed in neoblasts.[45,46] Key protein components of miRNA effector complexes (RISC) are members of the Argonaute family. Interestingly, two genes encoding members of the Argonaute family are expressed in neoblasts and knockdown of mRNAs encoding one of these proteins (*smedwi-2*) blocked regeneration.[43] It is not yet clear what small RNAs are affected by loss of *smedwi-2*, whether miRNAs or a closely related set of small RNAs referred to as piwi-associated RNAs (piRNAs),[42] but the findings are consistent with a key role for small RNAs in controlling stem cell gene expression pathways. In this case, the loss of *smedwi-2* did not alter neoblast maintenance but affected one or more steps of differentiation. By identifying the target genes controlled by these and other small RNAs, the hope is that key genetic pathways can be identified and dissected to potentially facilitate regeneration in higher organisms and/or to possibly allow reprogramming of adult stem cells.

miRNAs Regulate Fin Regeneration

Zebrafish are able to regenerate many damaged tissues and organs including fins, hearts, retinas and spinal cords.[47] Zebrafish caudal fins are relatively simple, composed of bony rays consisting of two hemirays that create a protective shell around nerves, blood vessels and mesenchymal cells. Fin regeneration involves five steps: wound healing, mesenchymal disorganization or reorganization, blastema formation, outgrowth and termination (Fig. 2). The key is the formation of a blastema, a structure containing stem cell-like cells that are either recruited to the damaged area or originate from the de-differentiation of cells in the area. How fin amputation sets in motion the five steps above and results in the formation of blastema cells remains largely unknown.

Recently, miRNAs have been shown to play a role in regulating fin regeneration in fish and tail regeneration in salamanders.[23,41,48] We showed that an intact miRNA biogenesis pathway is required for caudal fin regeneration in zebrafish; inhibition of Dicer by injection of anti-sense morpholinos blocked regeneration.[23] Given that miRNA expression patterns change, both up and down, during regeneration, it appears that overall gene regulation

requires proper control of both positive and negative regulators of regeneration. Consistent with this, some of the miRNAs that get downregulated during regeneration, including *miR-203* and *miR-133,* are predicted to target genes that are upregulated during regeneration (*lef1, msxb, fgfr1, fgf20a and hsp60).*[23,48] Conversely, a subset of miRNAs whose expression levels increase are predicted to target genes that are downregulated during regeneration.[23] One example is *miR-200b* which increases during regeneration. One of its predicted targets, *bmp3,* is correspondingly downregulated during regeneration.[49] More broadly, genes that are downregulated during regeneration are likely involved in preserving the terminally differentiated state and are therefore normally tightly controlled, reflecting the balance between growth and possible out of control proliferation.

Another interesting aspect of recent discoveries involving miRNAs during fin regeneration is that the first two identified miRNAs regulate key players involved in Wnt and Fgf signaling.[23,48] One of the earliest events upon damage is wound healing which results in early activation of Fgf signaling cascades, perhaps activated by hydrogen peroxide synthesis at the wound site.[50] Downstream, or coincident with Fgf signaling, a number of signaling cascades are activated including wnts, hedgehogs and TGFβ.[51] One possible role for miRNA regulation is to ensure that the gradients or readouts formed by these signaling cascades are properly initiated, maintained and finally, terminated. In many ways, this may be similar to such regulation during normal development, such as the role of *miR-214* to control muscle differentiation.[1]

miRNAs Regulate Muscle Regeneration

miRNAs play key roles in muscle, controlling specification, proliferation and differentiation.[52] *miR-1* and *miR-133* are expressed in cardiac and skeletal muscle where they are transcriptionally regulated by the well known myogenic factors MyoD, myogenin, Mef2 and SRF (serum response factor).[53–57] Expression of *miR-1* in mammals terminates the cell cycle in cardiac and skeletal muscle progenitors and promotes terminal differentiation.[54,57,58] *miR-133* has the opposite effect to maintain proliferating progenitor cells.[53] *miR-206* is expressed only in skeletal muscles and is induced by MyoD and myogenin to promote muscle differentiation.[59,60] This is consistent with the notion that specific subsets of miRNAs are expressed that can act in opposition to either promote or repress renewal versus differentiation (Fig. 3).

Pax3 and Pax7 are known to be expressed in skeletal muscle stem cells and represent a self-renewing population of cells that are critical for muscle growth and to maintain satellite cells that are required for the regeneration of adult skeletal muscle.[61] Pax3 is essential for maintenance of these cells and its migration to sites of myogenic differentiation and is regulated by *miR-27b* to rapidly initiate terminal muscle differentiation.[62]

miRNAs in Pancreas/β-cell Regeneration

Three major pancreatic transcription factors are necessary for early development, FoxA2, Hes1 and Pdx1.[63–65] Conditional Dicer deletion in pancreatic progenitor cells adversely affects the development of β-, δ-, exocrine and duct cells, indicating that proper miRNA-mediated gene regulation is critical for normal pancreas development, glucose metabolism

and insulin secretion.[66] Consistent with this, *miR-124a* downregulates *foxa2* expression which in turn regulates the targets of FoxA2 including *pdx1, kr6.2, sur-1*.[64]

Several groups have shown that NGN3 is a "master-controller" of pancreas development.[63,67–70] Hairy/enhancer of split (*hes1*), a notch signaling effector, tightly controls the number of Neurogenin3 (NGN-3) producing cells[65] and knockout of *hes1* can lead to ectopic pancreas development. miRNA profiling of the regenerating mouse pancreas has indicated that *miR-15a, -15b, -16 and -195* negatively regulate translation of *ngn3* (Fig. 3).[71] *miR-23* downregulates *hes1* during neuronal development and it may have a similar role in the pancreas.[72] In the regenerating pancreas, NGN3 levels are low, despite the fact that a number of activating transcription factors upstream of NGN3 are expressed. One possibility is that miRNA control of *ngn3* limits its expression. Interestingly, NGN3 is required to regenerate hormone-producing cells and it is possible that understanding how to alter NGN3 levels might help to treat diabetes through promoting regeneration of insulin-secreting cells.

Recent studies have shown a requirement for notch signaling in neuronal stem cell maintenance in embryonic and adult mice.[73] *Hes1;Hes3;Hes5* triple-mutant mice lack neural stem cell populations in most of the central nervous system except the telencephalon.[74] All other neuronal progenitor pools prematurely differentiated into neurons. In order to more completely deplete Notch signaling in the adult mouse brain, the Kageyama group knock-downed Rbpj in mice. The Notch intracellular domain (NICD) forms a complex with Rbpj to induce the expression of transcriptional repressors like Hes1.[73] Without repression of neuronal genes, cells prematurely differentiate. Thus, proper Notch signaling is crucial to regulate and maintain stem cell populations. miRNAs are likely to play a role in properly regulating Notch signaling, consistent with the finding that a number of miRNAs exhibit altered expression patterns upon inhibition of Notch.[19]

Initiation and Termination of Regeneration

The parallels to regeneration and cancer or tumor formation are many. As an example, during fin regeneration, genes that maintain the differentiated state must be turned off while genes that promote proliferation and outgrowth must be turned on. At the end of regeneration, the regulatory switches need to be reversed. It is remarkable that zebrafish fin regeneration terminates at precisely the right size, despite the fact that fish grow throughout their lifetimes. This raises the question as to what mechanisms are in place to properly terminate regeneration. We have shown that continued repression of *miR-203* during fin regeneration leads to fin overgrowth.[23] It remains unknown what signals regulate the transcriptional activity of *miR-203* but it is clear that specific miRNAs are needed for both initiation and termination. For unregulated growth in cancer, the similarities are striking. Overexpression of *miR-34* induces apoptosis and inhibits cell growth and tumor invasion in the pancreas via targeting of bcl-2 (B-cell CLL/lymphoma-2) and Notch1/2.[75–77] In pancreatic cancer, the action of *miR-34* must be downregulated to escape such growth controls.

Conclusion

The processes and mechanisms that allow certain organisms to undergo regeneration of complex tissues are only just beginning to be understood. The key lies in understanding those mechanisms (Fig. 3) and especially the role that stem cells play in these processes. As more and more is learned about regeneration, key regulatory steps that control stem cells and regeneration will be uncovered opening the door to application in humans to repair injured or damaged tissues.

Abbreviations

|	
miRNAs	microRNAs
RISC	RNA induced silencing complex
NGN3	neurogenin3
ES cells	embryonic stem cells

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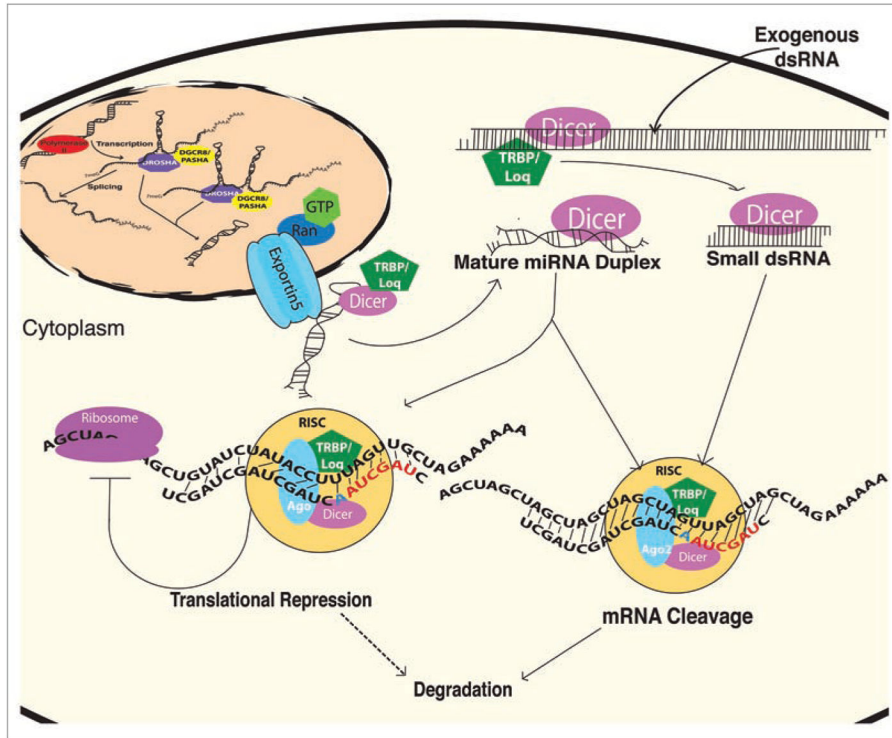
Figure 1.
miRNA Biogenesis. Long mono- and poly-cistronic miRNA primary transcripts are synthesized by RNA Polymerase II. These transcripts are processed in the nucleus by Drosha and exported in a Ran-GTP dependent process to the cytoplasm. Dicer recognizes cytoplasmic hairpin precursors, cleaves the loop regions, and mature miRNAs are incorporated into RISC complexes. RISC guides the miRNA to its target 3′UTRs. If the miRNA binds with perfect complementarity, mRNA cleavage occurs. If the miRNA binds with partial complementarity, translation repression occurs with possible subsequent degradation. siRNAs utilize the same cytoplasmic pathway to silence RNAs after delivery of exogenous double stranded RNAs (dsRNAs).
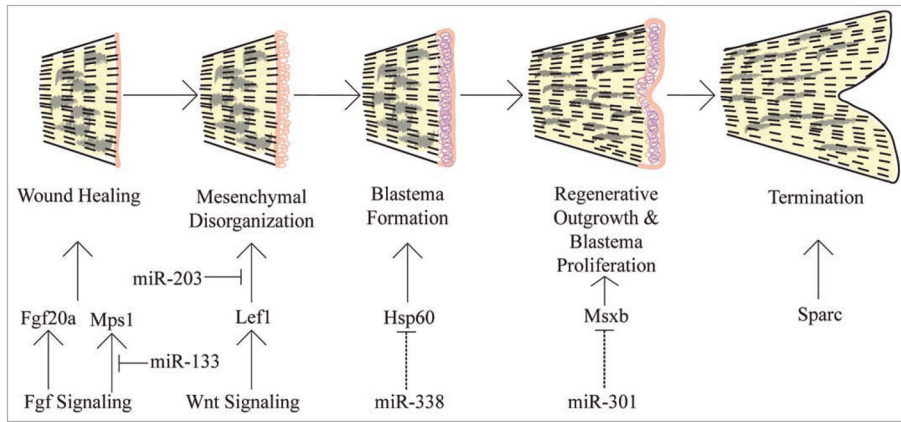
Figure 2.
Stages of zebrafish fin regeneration. Five stages of zebrafish fin regeneration are illustrated. Initial wound healing responses apparently stimulate a number of signaling pathways that ultimately result in the formation of a blastema, a structure that contains stem cell-like cells which divide and differentiate to re-form complex tissues. miRNA expression analyses have shown that *miR-203* and *miR-133* regulate targets as part of the Fgf and Wnt cascades,[23,48] other miRNAs are predicted to regulate downstream targets (dashed lines[23]).
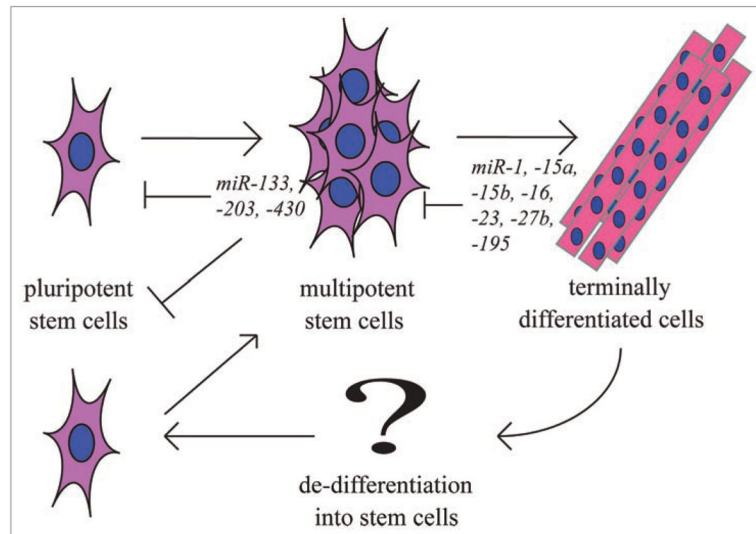
Figure 3.
Stem cell maturation. During normal development and during regeneration, stem cells play key roles in self-renewal, differentiation and specification. Shown are several miRNAs that have been implicated in the maintenance and differentiation of stem cells.