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Small RNAs: Keeping Stem Cells in Line

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Stem cells and RNA silencing have emerged as areas of intense interest for both basic and clinical research. Recently these fields have converged with reports implicating small regulatory RNAs in the maintenance and pluripotency of stem cells.

Metazoans rely on reservoirs of multipotent stem cells to not only initiate organismal development but also maintain tissues during adulthood. These programmable stem cells represent a state of cellular potential from which informed development can proceed. The qualifications for "stemness" include the ability of the undifferentiated cell to self-renew as well as the capacity to beget differentiated cell types. From this rubric, three classes of stem cells emerge: pluripotent embryonic stem cells, adult stem cells of somatic tissues, and adult stem cells of the germline. The instructions governing the differentiation of stem cells include intrinsic signals in the form of epigenetic programming and gene expression and external signals from the surrounding cellular environment, or niche.

Elucidation of RNA-silencing phenomena has implicated RNA-based modes of gene regulation as important mediators of stem cell maintenance and differentiation. In this Minireview, we focus on those small RNA classes with demonstrated roles in metazoan stem cells—microRNAs (miRNAs) and Piwi-interacting RNAs (piRNAs).

The RNA Revolution Meets Stem Cells

Originally characterized in genetic screens for factors affecting developmental timing in the worm *Caenorhabditis elegans*, miRNAs have been found to modulate gene expression in a variety of developmental settings. These endogenously expressed RNAs are transcribed in the nucleus and are sequentially processed by the RNaseIII enzymes, Drosha and Dicer, into small (~21 nucleotide) RNAs. The small RNAs are then further loaded into an RNA-induced silencing complex (RISC), containing a member of the Ago clade of the Argonaute protein family. These miRNA-directed ribonucleoprotein complexes regulate the expression of mRNAs bearing partially complementary "target sites" in their 3′untranslated region (UTR). More recently, a new class of small RNAs, piRNAs, has been identified in the germline of several animals. These noncoding RNAs are typically longer (24–31 nucleotides) than miRNAs and are generated in a Dicer-independent fashion. Instead, their biogenesis appears to depend upon the action of members of the Piwi subfamily of Argonaute proteins.

Regulation of gene expression by small RNAs is a dynamic field of study, with cellular and developmental functions being ascribed at a rapid pace. Loss-of-function studies targeting RNA-silencing components clearly implicate small RNA pathways in cell division, maintenance, and differentiation of stem cells. Here we discuss the emerging roles of these small RNAs in the various stem cell populations of developing animals (Figure 1).

Embryonic Stem Cells: More than Two Hundred Roads Diverged in a Wood…

The multicellular blastocyst formed during early embryonic development contains an inner cell mass from which pluripotent embryonic stem (ES) cells are derived (see Essay by J. Rossant and Review by C.E. Murry and G. Keller in this issue of *Cell*). These highly plastic cells can give rise to all three germ layers of the developing animal, and they can be cultured and studied ex vivo. This population of stem cells has captured the imagination of biologists and clinicians alike, as they bear the potential to develop into nearly all cell types of the animal.

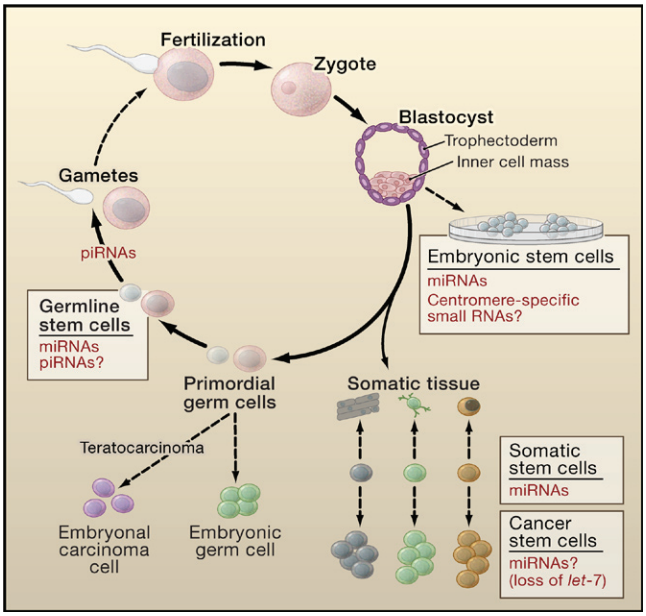

Figure 1. Small RNAs in Stem Cell Populations
Mammalian stem cells include embryonic stem cells derived from the inner cell mass of the blastocyst and adult stem cells of the somatic tissues and germline. Embryonic germ cells derived by ex vivo culture of primordial germ cells and embryonal carcinoma cells isolated from teratocarcinomas display similar hallmarks of stem cell pluripotency. Additionally, cancer stem cells are proposed to arise from oncogenic events in adult stem cells.

Genetic studies of various components of RNA silencing in several model organisms have demonstrated the crucial roles of these silencing pathways in both early embryonic development and ES cells. In the most obvious instances, genetic ablation of an RNA-silencing factor induces embryonic lethality. In fruit flies, homozygous mutation of the miRNA-processing enzyme Dicer-1 is lethal, and in mice, disruption of the single Dicer protein leads to embryonic lethality (Kloosterman and Plasterk, 2006). The defective embryos of the Dicer mutant mice display gross morphological abnormalities, and in situ hybridization demonstrated that the embryos have greatly reduced Oct4 expression, implying a lack of stem cells.

Although these studies demonstrated the importance of RNA-silencing components in embryonic development, more defined roles for small RNAs in ES cells have been observed through the use of conditional gene targeting. Disruption of Dicer function in murine ES cells produced several interesting findings (Kanellopoulou et al., 2005; Murchison et al., 2005). These mutant cells were defective in miRNA processing and greatly impaired in their ability to differentiate. Somewhat surprisingly, the Dicer-deficient ES cells were able to divide and form characteristic ES cell colonies, albeit with slower growth kinetics than their wild-type counterparts. Also, centromeric repeat sequences were expressed at higher levels in the mutant cells, and a fraction of small RNAs corresponding to these repeat sequences were curiously absent in these cells, suggesting that they may be involved in regulating the expression of these heterochromatic sequences. Intriguingly, the increased expression of these repeat sequences occurred in concert with changes in the formation of heterochromatin, raising the possibility that the Dicer-derived, centromeric repeat-specific small RNAs may be involved in transcriptional gene silencing via heterochromatin formation, as has been described in other organisms (Seto et al., 2007). In another conditional gene targeting study, DGCR8, a double-stranded RNA (dsRNA)-binding protein that forms the nuclear miRNA-processing complex (microprocessor) along with Drosha, was disrupted in murine ES cells (Wang et al., 2007). Like the ES cells lacking Dicer, the DGCR8 mutant cells were able to proliferate and form colonies but were deficient in their abilities to differentiate. However, both the proliferation and differentiation defects were not as pronounced as those observed in the Dicer knockout ES cells, suggesting that some miRNAs might bypass Drosha processing and/or that Dicer could have pleiotropic roles in ES cells.

These studies provide strong evidence that miRNAs play critical roles in the ability of ES cells to differentiate. Though not yet formally demonstrated, these studies imply that miRNA-based regulation of factors that specify stem cells is crucial for differentiation. Several groups have undertaken large-scale cloning and sequencing efforts to delineate the miRNA profile in several human and murine ES cell lines (Calabrese et al., 2007; Suh et al., 2004). These studies demonstrated that the ES cells express a unique repertoire of miRNAs (including several miRNA families conserved in humans and mice), whose expression appears to be enriched in these cells. Interestingly, one of these ES cell-enriched miRNA families in human ES cells, the miR-302 family, is orthologous to the zebrafish miR-430 family, whose expression is critical for early development (Giraldez et al., 2006). High-throughput sequencing analysis allowed for rigorous evaluation of miRNA expression in murine ES cells, and these data demonstrated that six miRNA loci accounted for ~70%–75% of the miRNAs detected in the cell lines. Several of these miRNA clusters have previously been implicated in oncogenesis and cell-cycle control, indicating that miRNAs likely play a critical role in regulating the cell cycle in ES cells. Additionally, the authors found no evidence of Dicer-dependent, repeat-associated small RNAs, as small RNAs found to map to repeat regions of the genome were detected in both wild-type and Dicer-deficient ES cells in their study. However, the particular Dicer mutant cell line used did not recapitulate the previously reported loss of DNA methylation at various genomic repeat loci (Kanellopoulou et al., 2005). A resolution of these issues is required to assess the functions, if any, for small RNA groups other than miRNAs in ES cell biology. Additionally, functional studies of miRNAs enriched in ES cells are necessary to reveal their contribution to stem cell biology.

Somatic Stem Cells: Out with the Old, in with the New

Although somatic stem cells have been described in numerous animals, they are often less well-defined than stem cells of the embryo or germline. Many mature tissues and organs have been found to contain populations of "stem cells" that give rise to multiple cells of that tissue. However, in the soma, the line between pluripotent self-renewing stem cells and uni- or multipotent progenitor cells is often blurred due to the difficulty in isolating these cell types and ascertaining their self-renewal and multipotency capabilities.

The conditional Dicer knockout system has provided insights into the function of small RNAs in somatic cells of specific tissues. For example, targeted deletion of Dicer in the limb mesoderm of the mouse embryo resulted in developmental defects and the formation of a smaller limb (Kloosterman and Plasterk, 2006). Although this and other similar studies have demonstrated roles for small RNAs in developing tissue and progenitor cell populations, they do not demonstrate a specific role in stem cells. A similar conditional knockout strategy was used to specifically disrupt the expression of Dicer in the epidermis of developing mice (Kloosterman and Plasterk, 2006). These mice displayed altered morphogenesis of hair follicles, and importantly, these follicles did not express stem cell markers and were not maintained. Further evidence for a role of small RNAs in somatic stem cells has been provided by a mosaic study in *Drosophila* in which Dicer was deleted specifically from ovarian somatic stem cells, and maintenance of these cells was lost (Jin and Xie, 2007).

Demonstrating roles for specific miRNAs in stem cells has proven elusive. This may be due to the fact that numerous miRNAs are members of paralogous families, which could provide functional redundancy. Also, it is now widely accepted that miRNA-based regulation depends on the coordinated efforts of multiple miRNAs to "fine-tune" gene expression. However, careful genetic studies to manipulate the expression of specific miRNAs during development and

functional studies in purified cell culture models have provided further clues for the involvement of miRNAs in progenitor cells and stem cells of the soma. Examination of a cardiac-enriched miRNA family indicated critical roles for these miRNAs in differentiation and proliferation of progenitor cells in the heart (Zhao et al., 2005). Additionally, experiments using isolated populations of hematopoietic stem cells have demonstrated roles for specific miRNAs in lineage differentiation, and evidence suggests that miRNAs are important for differentiation of somatic stem cells in several other tissues (Lakshmipathy and Hart, 2007). Interestingly, a recent study in a purified mammary epithelial cell line indicated that the presence or absence of specific miRNAs may be used as a marker to enrich for self-renewing progenitor or stem cell populations (Ibarra et al., 2007).

Germline Stem Cells: Fountains of Youth

A third group of metazoan stem cells, germline stem cells (GSCs), are descendants of the primordial germ cells (PGCs), which are formed early in embryogenesis (see Minireview by R.M. Cinalli et al. in this issue). Following specification of the PGCs, these cells migrate to the gonad, where they form the GSCs. The GSCs beget the germ cells, which undergo gametogenesis to produce mature eggs and sperm.

Numerous intrinsic and niche-produced extrinsic factors have been shown to affect the maintenance of germline cells (see Review by S.J. Morrison and A.C. Spradling in this issue). Notably, genetic studies in several species have clearly demonstrated an important role for miRNA-silencing mechanisms in gametogenesis. Loss of Dicer in *C. elegans* results in impaired germline maintenance and sterility (Knight and Bass, 2001). Analysis of *Drosophila* GSCs reveals two cell-autonomous functions for miRNAs—regulation of GSC division and maintenance (Forstemann et al., 2005; Hatfield et al., 2005; Jin and Xie, 2007; Park et al., 2007; Shcherbata et al., 2007; Yang et al., 2007). One molecular target of miRNA regulation during division of fly GSCs is the sole cyclin-dependent kinase inhibitor, Dacapo, a p21/p27 homolog. Regulation of fly GSC maintenance by miRNAs is less well understood, although interestingly, young GSCs can compensate for miRNA defects, a capacity that is lost as GSCs age. Furthermore, these studies have also indicated that an miRNA called *bantam* is required cell autonomously in adult stem cells (Shcherbata et al., 2007).

Taken together, these invertebrate studies imply that miRNAs are required not only for gametogenesis but also for normal GSC maintenance and control of cell division. Targeted disruption of Dicer in the female germline of mice also results in impaired gametogenesis, and the data further suggest that transposon-derived small RNAs produced by Dicer contribute to clearance of maternally derived RNAs in oocytes (Murchison et al., 2007). This supports findings in zebrafish where small RNAs are involved in clearance of maternal RNAs (Giraldez et al., 2006). However, it remains to be determined if Dicer is required specifically in mammalian GSCs, and whether these repeat-associated small RNAs that arise in the germline function in GSCs or only in meiotic stages of developing germ cells.

Genetic studies have implicated the involvement of another group of RNA silencing-associated factors in germline stem cell function. In fruit flies, mice, and zebrafish, multiple members of the Piwi clade of the Argonaute protein family are involved in germline maintenance (Aravin et al., 2007; Seto et al., 2007). Mutant *piwi* fruit flies undergo loss of GSCs in both males and females, although loss of *piwi* expression in the soma contributes to this phenotype. The loss of *piwi* in the germline alone affects GSC division but not maintenance. In zebrafish, *ziwi* mutants experience loss of germ cells in both males and females, whereas deletion of *Miwi2* in mice leads to germ cell loss only in the male germline. Furthermore, defects in gametogenesis have been described for numerous other Piwi family members when mutated, including *Mili* and *Miwi* in mice, *aubergine* in flies, and *prg-1* and *prg-2* in worms. These findings suggest that different Piwi family members are involved at multiple stages of the meiotic and mitotic divisions that characterize gametogenesis (Seto et al., 2007). Further studies are required to define which Piwi-class proteins, if any, are required cell autonomously in GSCs.

A flurry of recent studies identified piRNAs as a new class of small RNAs, which associate with Piwi proteins. Initially identified in flies, Piwi- and Aubergine-associated small RNAs are enriched for repetitive elements in heterochromatic regions of the genome. These repeat-associated small interfering RNAs (rasiRNAs) are considered a subclass of piRNAs; loss of some rasiRNA-generating loci or mutations in *piwi* or *aubergine* result in mobilization of transposons (Aravin et al., 2007; Seto et al., 2007). More recently, piRNAs were isolated from mouse and rat testes (Aravin et al., 2007; Seto et al., 2007). piRNAs are not generated by Dicer and do not appear to originate from dsRNA or miRNA precursor-like structures. Instead, it has been postulated that these poorly conserved small RNAs are generated through an amplification cycle of piRNA-containing transcript cleavage events mediated by Piwi proteins (Aravin et al., 2007).

For most animals, production of mature gametes from GSCs is a multistage process involving both mitotic and meiotic divisions. The accumulating evidence of RNA-silencing mechanisms in the germline suggests that small RNAs play key roles in regulating GSC development and in maintaining genomic integrity in the diploid and haploid cells resulting from this process.

Future Perspectives

Stem cell research offers the considerable rewards of furthering our understanding of developmental biology as well as providing the attractive possibility of cell-based therapies. The study of RNA-silencing mechanisms similarly provides important information regarding development in addition to providing potential avenues for gene-specific therapeutics. The emerging intersection of these two fields is not altogether surprising given that the development and maintenance of tissues are intricately choreographed processes requiring stem cell populations to rapidly respond to multiple organismal and environmental cues. The task of such regulation is well suited to small RNAs, like miRNAs, which can rapidly dampen levels

of gene expression. Additionally, piRNAs appear to serve an important role as protectors of the genome in the germline, allowing reliable production of mature gametes from the germline stem cells.

Might RNA-silencing pathways play additional roles in stem cell biology? An extremely interesting example of such a case has been observed in the planarian *Schmidtea mediterranea*. These flatworms are capable of regenerating organs, as well as regenerating entire individuals from small portions of their body (see Review by K.D. Birnbaum and A. Sánchez Alvarado in this issue). The ability to accomplish these feats lies in the neoblasts, a population of somatic stem cells, which are also the only mitotic cells in the adult animal. An RNAi screen for factors involved in regeneration implicated a Piwi-family protein, Swedwi-2, in the differentiation and renewal of neoblasts in response to wounding. With the speed at which research is progressing in stem cell biology, additional roles for RNA-silencing mechanisms are likely to emerge.

Finally, we must consider the potential involvement of RNA-silencing pathways in stem cell malignancies. Although we have espoused the virtues of the flexible and sensitive level of gene (and genomic) regulation afforded by small RNAs, it must be pointed out that aberrancies in these small RNAs or silencing pathways may also contribute to disease. Indeed, not long after the characterization of miRNAs, reports surfaced correlating misexpression or loss of miRNA loci with various tumors (Kloosterman and Plasterk, 2006). In recent years, evidence for "cancer stem cells" has accumulated. Many types of tumors contain a stem cell population that is proposed to propagate the growth and metastasis of the tumor. Intriguingly, miRNA profiling of human and mouse ES cells reveals high levels of expression of miRNAs previously associated with oncogenesis and cell-cycle control (Calabrese et al., 2007; Suh et al., 2004). Additionally, just as lack of *let-7* miRNA expression was observed as an indicator for "stemness" in epithelial progenitor cells, recent studies have demonstrated that *let-7* expression is absent from certain tumor cell lines, and that reintroduction of *let-7* into these cells causes differentiation and reduction in proliferation and tumor-forming ability (Figure1) (Ibarra et al., 2007; Yu et al., 2007). Clearly, small RNAs are likely to be instrumental in helping to control the delicate balance between the extraordinary ability of stem cells to self-renew and differentiate for the purposes of development and tissue maintenance versus their potential for dysregulated growth and tumor formation.

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