MINIREVIEW

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Piecing Together the Mosaic of Early Mammalian Development through MicroRNAs*

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The microRNA (miRNA) pathway represents an integral component of the gene regulation circuitry that controls development. In recent years, the role of miRNAs in embryonic stem (ES) cells and mammalian embryogenesis has begun to be explored. A few dozens of miRNAs expressed in mammalian ES cells, either exclusively or nonexclusively, have been cloned. The overall role of miRNAs in ES cells and embryonic development has been assessed by examining the effect of knocking out Dicer, an RNase III enzyme required for miRNA and small interfering RNA biogenesis, as well as DGCR8, a nuclear protein specifically involved in miRNA biogenesis. In addition, the role of a cluster of miRNAs specifically expressed in ES cells, the miR-290–295 group, has been investigated by the knock-out approach. These analyses have revealed the crucial role of miRNAs in ES cell differentiation, lineage specification, and organogenesis, especially neurogenesis and cardiogenesis. Systematic investigation of the role of miRNAs in ES cells and embryos will allow us to find missing pieces of the mosaic of early development.

Embryonic stem (ES)[2] cells, derived from the inner cell mass of mammalian blastocysts, are pluripotent cells that have the ability to differentiate into all three germ layers of the embryo: endoderm, mesoderm, and ectoderm. As a result of their plasticity and self-renewing ability, ES cells possess compelling therapeutic potential in the regeneration of tissues and the treatment of disease. At present, molecular mechanisms underlying ES cell pluripotency and self-renewal are being revealed at an exciting rate.

The emergence of microRNAs (miRNAs) as potent regulators of gene expression at the post-transcriptional level has broad implications in all facets of biology, including ES cells and early development. Computational algorithms suggest that the ~20–22-nucleotide miRNAs have the capability of binding to a plethora of mRNAs (1, 2). Therefore, the gregarious nature of miRNAs impeccably suits them for participation in regulating differentiation and development via global, rapid, and coordinated changes in protein expression. Each miRNA espouses the possibility of being a missing piece of the mosaic of early development (a *tessera*), which will fill interstices of the intricate mosaic and generate a more refined and unmitigated picture of early embryonic development. In this review, we discuss recent progress in understanding the role that miRNAs play in mammalian ES cells and early development.

Expression of miRNAs in Mammalian ES Cells

Hundreds of miRNAs have been cloned from mammalian cell lines and organs. Two studies have reported that some miRNAs are specifically expressed in ES cells, thus implicating miRNAs in ES cell self-renewal, pluripotency, and early mammalian development.

In 2003, Houbaviy *et al.* (6) cloned ES cell-specific miRNAs from three miRNA libraries constructed from mouse ES (mES) cells. Library 1 (L1) was from mES cells grown on a feeder layer of mouse embryonic fibroblasts (MEFs) and leukemia inhibitory factor (LIF). LIF maintains the self-renewal of mES cells (3). Expectedly, this library represents the highest fraction of undifferentiated ES cells. Library 2 (L2) was from ES cells grown in the presence of LIF but without a feeder layer. This library consequently represents miRNAs from undifferentiated ES cells and is free of potential contamination of MEF-derived miRNAs. Finally, Library 3 (L3) was from mES cells grown in the presence of retinoic acid but without LIF. This library represents a subset of miRNAs induced during mES cell differentiation. The degree of differentiation in these libraries was evaluated by the steady-state levels of *Oct4* mRNA, a transcription factor that is down-regulated during differentiation of mouse and human ES cells (4) and in the presence of alternatively spliced isoforms of α6-integrin mRNA, with the shorter form indicating the undifferentiated state (5). In concert with normally observed mES cell differentiation patterns, the L1 and L2 libraries had the highest expression levels of *Oct4* mRNA and the short α6-integrin mRNA isoform (6). In contrast, the L3 library showed a 5-fold reduction in *Oct4* mRNA levels and expression of the long α6-integrin isoform (6).

Using the aforementioned miRNA ES cell populations, Houbaviy *et al.* (6) cloned a total of 53 miRNAs. These miRNAs fit the criteria of being 20–24 nucleotide in length, folding into potential pre-miRNA hairpin precursors, and having a phylogenetically conserved hairpin fold. Fifteen of the clones were novel miRNAs that were unrelated to any previously isolated and cloned miRNAs. Furthermore, sequence alignment analyses showed that six pre-miRNA precursors (miR-290–295) share a 23-nucleotide nearly identical sequence. This cluster of miRNAs also mapped to a 2.2-kb region of the mouse genome. Interestingly, the 15 miRNAs are also poorly conserved in other genomes (6), suggesting a species-specific role for these miRNAs.

Houbaviy *et al.* (6) proposed that the group of related miRNAs (miR-290–295) is potentially ES cell-specific and could have ES-specific functions, such as maintaining pluripotency. This hypothesis was supported by three lines of observations.

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[2] The abbreviations used are: ES, embryonic stem; miRNAs, microRNAs; mES, mouse ES; MEFs, mouse embryonic fibroblasts; LIF, leukemia inhibitory factor; hES, human ES; siRNA, small interfering RNA.

First, these miRNAs are novel miRNAs that have not been observed previously. Second, these miRNAs are silenced or down-regulated during EC cell differentiation. Their expression is not detectable in MEFs, embryoid bodies, or adult organs. Although these miRNAs are expressed in the L1, L2, and, unexpectedly, also L3 libraries (6), the expression in L3 might be due to their long half-life. Third, express sequence tag data base searches for entries homologous to the 2.2-kb genomic sequence that contains the cluster of the six pre-miRNAs identified cDNAs from pre-implantation embryos or ES cells, further implicating these miRNAs to be ES cell-specific.

In a similar study, Suh *et al.* (7) isolated >36 miRNAs from human ES (hES) cells, most of which are specifically expressed in hES cells but not in differentiated embryonic cells or adult tissues. Among them, 17 had not been described previously, and three (miR-296, miR-301, and miR-302) are identical to the mES cell-specific miRNAs reported by Houbaviy *et al.* (6). One miRNA (miR-302) is specific to mES cells, hES cells, and human embryonic carcinoma cells. Interestingly, genomic loci for some of these miRNAs are organized into two clusters (7). For example, eight miRNA loci (miR-302, -302b*, -302c, -302c*, -302a, -302a*, -302d, and -367) are within a 700-bp region on chromosome 4. Some of the miRNAs on the chromosome 4 cluster appear to be the human homologs of mouse miR-302 (6). Four other loci (miR-371, -372, -373, and -373*) are clustered within a 1050-bp region on chromosome 19. The chromosome 19 cluster represents the human homolog of the mouse miR290–295 cluster that is expressed in mES cells (7). Furthermore, miRNAs in these clusters appear to be cotranscribed as polycistronic transcripts (7). The clustered organization of these ES cell-specific miRNAs, in which multiple miRNAs share a common promoter and a common precursor transcript, is presumably effective for coordinated regulation of their coordinated expression in ES cells. In support of this notion, both the chromosome 4 and 19 clusters are rapidly down-regulated or silenced upon differentiation of ES cells to embryoid bodies (7). In particular, the expression profile of the chromosome 19 cluster is conspicuously pertinent because the down-regulation of this cluster occurs prior to the down-regulation of *Oct4* mRNA, one of the earliest markers of ES cells (8, 9). Therefore, the chromosome 19 cluster miRNAs appear to be earlier markers of ES cells than *Oct4* and may have functions in maintaining ES cell pluripotency.

Although the two hES cell-specific miRNA clusters are conserved in the mouse genome, many miRNAs cloned in mES and hES cells appear to be poorly conserved (7). This could be due to the incomplete coverage of the cloning efforts, different developmental repertoires among different species, or both. Differences aside, the studies by Houbaviy *et al.* (6) and Suh *et al.* (7) suggest that the mammalian miRNAs are likely involved in ES cell pluripotency and/or early embryogenesis in mammals. The precise mechanism of this involvement awaits further investigation.

Function of miRNAs in ES Cells and Early Embryogenesis

The global function of miRNAs in ES cells and early embryogenesis is now being revealed by loss-of-function analysis of miRNA clusters and key proteins in the miRNA biogenesis pathway.

Role of miR-290–295 Group miRNAs in Development—Recently, Sharp and co-workers (6) generated a mouse strain in which the ES cell-specific miR-290–295 group of miRNAs is deleted. The homozygous mutant animals mostly die as embryos; only a few manage to grow into adulthood. This indicates the important role of these miRNAs in embryogenesis. Of note, female mutant survivors are infertile. This observation delineates a function of miRNAs not only in maintaining pluripotency but also in female fertility (10).

Role of Dicer in ES Cells—The overall role of miRNA in ES cells and early embryogenesis can be assessed by analyzing mice that are deficient in Dicer, an RNase III enzyme required for miRNA and small interfering RNA (siRNA) biogenesis. Dicer cleaves double-stranded RNA or miRNA precursors into mature siRNAs or miRNAs in the cytoplasm (1). The role of Dicer in the development of different organisms has been extensively studied. In *Drosophila*, two Dicer isozymes exist: Dicer-1 (DCR-1) is required for the miRNA pathway, whereas Dicer-2 is required for the siRNA pathway (11). Work by Hatfield *et al.* (12) suggests that DCR-1 is required for normal germ line stem cell self-renewal in *Drosophila*. Mutant *Dcr-1* germ line stem cells exhibit a delayed G_1/S transition. In zebrafish, Dicer is also essential for normal embryonic development (13). Dicer knock-out and knockdown mutants show a lack of miRNA production and experience an overall arrest during embryogenesis (13).

In concurrence with *Drosophila* and zebrafish studies, Dicer in mice is also requisite for embryogenesis. Constitutive loss of DCR-1 leads to embryonic lethality (14). The essential role of miRNAs in mouse early development is further supported by conditional knock-out studies of Dicer in mES cells. Kanellopoulou *et al.* (15) successfully produced Dicer-null mES cells using a Cre-*loxP* method. These ES cells show an accumulation of miRNA precursors, but they lack the ability to produce mature miRNAs and siRNAs. These ES cells are viable, displaying morphology identical to their wild-type and heterozygous ES cell counterparts. However, they show reduced proliferation rate and little evidence of differentiation to embryoid bodies. In addition, markers of endodermal (*Hnf4*) and mesodermal (*brachyury*, *Bmp4*, and *Gata1*) differentiation are absent in these cells, whereas the pluripotent marker *Oct4* remains at a high level of expression after 5 days of attempted differentiation, in contrast to the wild-type situation (15). Moreover, Dicer-deficient cells show alterations in DNA methylation of centromeric repeats and histone modifications (15), revealing the role of Dicer, and possibly miRNAs, in the maintenance of mammalian heterochromatin structure and centromeric silencing during embryogenesis. The reintroduction of Dicer to the Dicer-deficient ES cells rescues the observed phenotype, validating the role of Dicer, at least partly via miRNAs, in ES cell differentiation (15).

In a separate study, Murchison *et al.* (16) found that Dicer-deficient ES cells proliferate more slowly, a defect that is rescued with time. They hypothesize that this rescue is due to the accumulation of secondary mutations. Moreover, these ES cells have increased G_1 and G_0 phases (16). It is possible that this phenotype is due to the loss of some miRNAs that regulate cell

cycle progression. Drawing a distinction from results reported by Kanellopoulou *et al.* (15), there is no apparent defect in DNA methylation in these Dicer-deficient ES cells. The inconsistency between the two studies could be explained by two possible reasons. First, the discrepant phenotype could be a result of the generation of a catalytically inert Dicer protein that preserves cytosine methylation at the centromeres (16). Or second, the differing results could be due to prolonged cell culture and consequent selective pressure, yielding two singular effects in methylation (16).

DGCR8 Knocking Out and Role of miRNA in ES Cells—Although the studies of Kanellopoulou *et al.* (15) and Murchison *et al.* (16) are consistent with the essential role of Dicer and miRNAs in ES cell proliferation and differentiation, the question remains whether the observed Dicer-deficient phenotype is a result of a defect in miRNAs or a synthetic phenotype due to the concurrent loss of miRNAs, siRNAs, or other unknown regulatory small RNAs. To definitively assess the role of miRNA in ES cells, Wang *et al.* (17) generated DGCR8 knock-out ES cells.

DGCR8 is a double-stranded RNA-binding protein that, in conjunction with the RNase III enzyme Drosha, constitutes the microprocessor complex necessary for the proper maturation of primary miRNAs to pre-miRNAs in the nucleus (1, 18). This nuclear processing by DGCR8 is ostensibly specific to miRNAs, in contrast to Dicer processing, which occurs in the cytoplasm and is involved in the siRNA maturation pathway as well (17). Thus, the phenotype of DGCR8 knock-out cells specifically reflects the loss of miRNA function.

DGCR8 knock-out mES cells show a phenotype that alludes to the global regulatory role that miRNAs play in ES cell proliferation, cell cycle progression, and differentiation. DGCR8 knock-out ES cells accumulate primary miRNA transcripts and lack mature miRNAs (17). These cells accumulate in the G_1 phase. They display an early arrest in development and an extended population doubling time in comparison with wild-type and heterozygous populations (17). These defects are reminiscent of the Dicer-deficient phenotype in mES cells as well as the *Drosophila* DCR-1 knock-out phenotype (12, 16). Although DGCR8-deficient cells are morphologically normal and express ES cell-specific markers, they show global defects in differentiation to embryoid bodies, lacking the ability to form cysts and having an abnormal expression profile of differentiation markers (*Fgf5*, *Hnf4*, and *Sox1*) (17). The reintroduction of DGCR8 into the knock-out cells rescues the observed phenotype, confirming DGCR8 as the cause of the defects (17). In the presence of retinoic acid, a more forceful inducer of differentiation, the typical markers of the pluripotent state in mES cells, such as *Oct4*, *Rex1*, *Sox2*, and *Nanog*, are not down-regulated, but some differentiation markers are expressed (17). These results diverge from the DCR-1 knock-out cells that do not express differentiation markers in embryoid body cultures (15). Moreover, although both DCR-1 knock-outs and DGCR8 knock-outs share proliferation defects, the DGCR8 knock-out cells seem to show a more stable defect that is not rescued by time (17). These observations together indicate that the miRNA pathway is essential for ES cell differentiation and that Dicer has a miRNA-independent function in promoting ES cell differentiation.

Function of miRNAs in Neurogenesis and Cardiogenesis

The role of miRNAs in organogenesis and lineage determination has become evident from recent studies in zebrafish and mammalian hematopoietic and adipocytic development. Moreover, these works have provided exciting insight into the role of miRNAs in neurogenesis and cardiogenesis.

Role of miRNA in Neurogenesis—In zebrafish, Giraldez *et al.* (19) showed that embryos depleted of both maternal and zygotic Dicer have normal early embryonic growth and axis formation; however, gastrulation, brain formation, somitogenesis, and heart development are defective. Surprisingly, the co-injection of miRNAs miR-430a, miR-430b, and miR-430c alone rescues the brain morphogenesis defect of the Dicer-depleted embryos (19). Follow-up studies demonstrated that these miRNAs accelerate the deadenylation and degradation of maternal mRNAs during the maternal-to-zygotic transition (20).

In mice, Krichevsky *et al.* (21) demonstrated the likely role of miRNAs in neural cell fate in mammalian brain development. The authors first used oligonucleotide arrays to show that miRNAs are specifically regulated during brain development. They then defined a role for miRNAs in ES cell-derived neurogenesis and differentiation (22). A five-step model of mES cell differentiation, including the ES cell stage, embryoid body stage, neural precursor stage, and neural differentiation stage, was used to mimic the corresponding steps of embryogenesis (22). miRNA microarray data showed a differential expression profile in these stages. Five miRNAs that were differentially expressed between the neural precursor and neural differentiation stages were chosen for further study (22). Among them, miR-124a and miR-9 are exclusively expressed in the brain (23). These two miRNAs show effects in ES cell-derived neurogenesis: their overexpression in neural precursors led to a 47–55% reduction of astrocytes (glial fibrillary acidic protein-positive cells). In addition, inhibition of miR-9, both alone and in combination with miR-124, yielded a reduction in the number of neurons (22). Thus, brain-specific miRNAs significantly affect the differentiation of ES cell-derived neural precursor cells along the glial and neuronal differentiation pathways.

Another brain-specific miRNA, miR-124, has also been implicated in neurogenesis. Visvanathan *et al.* (24) found that the down-regulation of the small C-terminal domain phosphatase gene *SCP1* during development is critical for neurogenesis. miR-124 targets the 3′-untranslated region of the *SCP1* gene and contributes to the down-regulation of SCP1 expression (24). This study provides additional evidence to the role of miRNAs in neural development.

Role of miRNA in Cardiogenesis—A recent study by Zhao *et al.* (25) has provided strong evidence for the *in vivo* requirement of miRNAs in normal heart development in mice. The authors first assessed the global function of miRNAs in cardiogenesis by deleting Dicer in the heart at embryonic day 8.5. As a result, the mutant embryos died from cardiac failure at embryonic day 12.5 (25). The authors then specifically deleted miR-1-2, which led to a range of phenotypes: ventricular septal defects, alterations in cardiac rhythm, and alterations in the cell cycle profile that leads to hyperplasia of the heart (25). These defects underscore the importance of miR-1-2 in cardiogenesis.

Conclusion

Stem cells and miRNAs represent two exciting new frontiers of biological research with far-reaching significance: one represents a fundamental biological question, and the other provides mechanistic insight. A fascinating intersecting point of these two frontiers is miRNAs in ES cells. Recent studies as reviewed here have demonstrated the important role of miRNAs in ES cell differentiation, lineage specification, and organogenesis. These studies also raise new questions. How many miRNAs are specifically expressed in ES cells? How many are nonspecifically expressed in ES cells? How do miRNAs modulate the expression of target mRNAs to regulate ES cells and early embryonic development? Answers to these questions will significantly advance our understanding of ES cell biology. Moreover, because the *in vitro* differentiation of ES cells mimics important events of early embryonic development, the combination of studies in ES cells and transgenic and knock-out models should allow us to effectively explore molecular and cellular mechanisms underlying embryogenic development.

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