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Small RNAs in early mammalian development: from gametes to gastrulation

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Summary

Small non-coding RNAs, including microRNAs (miRNAs), endogenous small interfering RNAs (endo-siRNAs) and Piwi-interacting RNAs (piRNAs), play essential roles in mammalian development. The function and timing of expression of these three classes of small RNAs differ greatly. piRNAs are expressed and play a crucial role during male gametogenesis, whereas endo-siRNAs are essential for oocyte meiosis. By contrast, miRNAs are ubiquitously expressed in somatic tissues and function throughout post-implantation development. Surprisingly, however, miRNAs are non-essential during pre-implantation embryonic development and their function is suppressed during oocyte meiosis. Here, we review the roles of small non-coding RNAs during the early stages of mammalian development, from gamete maturation through to gastrulation.

Key words: siRNA, MicroRNA, Stem cells, Oocyte, Embryonic development

Introduction

The processes of growth and differentiation are kept in balance during the development of multicellular organisms. Post-transcriptional control of gene expression plays a key role in this balance by coordinating the expression of selected genes at specific times and places. The role of post-transcriptional regulation is particularly apparent in early mammalian development, from maturation of the germ line to initiation of gastrulation, when controls on mRNA localization, stability and translation are the fundamental means of gene regulation. Indeed, from the fully grown oocyte stage until zygotic genome activation (ZGA), the genome is transcriptionally silent (Abe et al., 2010). Therefore, all mRNA regulation must occur post-transcriptionally. Following ZGA, the embryo establishes populations of early stem cells (SCs) within the inner cell mass (ICM, the collection of cells that eventually will form the fetus) that begin to proliferate rapidly, ensuring that stocks of unspecialized cells are established for future differentiation into the three germ layers. This rapid growth and the subsequent switch from unspecialized cells into specific cell types is a highly regulated process involving much post-transcriptional regulation (Lu et al., 2009). Similar regulation also occurs in the extra-embryonic tissues. This review will focus on one group of post-transcriptional regulators, the small non-coding RNAs. These RNAs range in size from 18 to 32 nucleotides (nt) in length and have emerged in the past decades as major players in post-transcriptional regulation across many, if not most, multicellular organisms.

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Classes and biogenesis of mammalian small RNAs

Three major classes of functional small non-coding RNAs have been found in mammals: microRNAs (miRNAs), endogenous small interfering RNAs (endo-siRNAs) and Piwi-interacting RNAs (piRNAs) (Babiarz and Blelloch, 2009; Kim et al., 2009; Thomson and Lin, 2009). These classes differ in their biogenesis, i.e. their maturation from transcribed forms to the active form of the RNA (Fig. 1).

miRNAs can be divided into two subclasses: canonical and non-canonical miRNAs. Canonical miRNAs are initially transcribed as long RNAs that contain hairpins (Fig. 1A). The 60-75 nt hairpins are recognized by the RNA-binding protein Dgcr8 (DiGeorge syndrome critical region 8), which directs the RNase III enzyme Drosha to cleave the base of the hairpin (Denli et al., 2004; Gregory et al., 2004; Han et al., 2004; Han et al., 2006; Landthaler et al., 2004; Lee et al., 2003). Following cleavage by the Drosha-Dgcr8 complex, also called the microprocessor, the released hairpin is transported to the cytoplasm, where Dicer, another RNase III enzyme, then cleaves it into a single short 18-25 nt dsRNA (Bernstein et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001; Knight and Bass, 2001). Non-canonical miRNAs bypass processing by the microprocessor by using other endonucleases or by direct transcription of a short hairpin. The resulting pre-miRNAs are then exported from the nucleus and cleaved once by Dicer (Babiarz et al., 2008; Okamura et al., 2007; Ruby et al., 2007).

By contrast, siRNAs are derived from long dsRNAs (Fig. 1B) in the form of either sense or antisense RNA pairs or as long hairpins, which are then directly processed by Dicer consecutively along the dsRNA to produce multiple siRNAs (Chung et al., 2008; Czech et al., 2008; Ghildiyal et al., 2008; Kawamura et al., 2008; Okamura et al., 2008a; Okamura et al., 2008b). Therefore, canonical miRNAs, non-canonical miRNAs and endo-siRNAs all involve Dicer processing and are ~21 nt in length. Furthermore, in all three cases, one strand of the Dicer product associates with an Argonaute protein (Ago 1-4 in mammals; also known as Eif2c1-4) to form the active RISC (RNA-induced silencing complex, Fig. 1D) (Filipowicz, 2005). These ribonucleoprotein complexes are able to bind to and control the levels and translation of their target mRNAs: if the match between the small RNA and its target is perfect, the target is cleaved; if not, the mRNA is destabilized through as yet unresolved mechanisms (Doench et al., 2003; Fabian et al., 2010; Zeng et al., 2003).

The piRNAs differ from the miRNAs and endo-siRNAs in that they do not require Dicer for their processing (Houwing et al., 2007; Vagin et al., 2006). Indeed, how piRNAs are produced and their mechanism of action remains poorly characterized (for a review, see Klattenhoff and Theurkauf, 2008). piRNAs are 25-32 nt in length, and are expressed predominantly in the germline in mammals (Aravin et al., 2006; Grivna et al., 2006; Watanabe et al., 2006). They are defined by their interaction with the Piwi proteins, a distinct family of Argonaute proteins (including Miwi, Miwi2

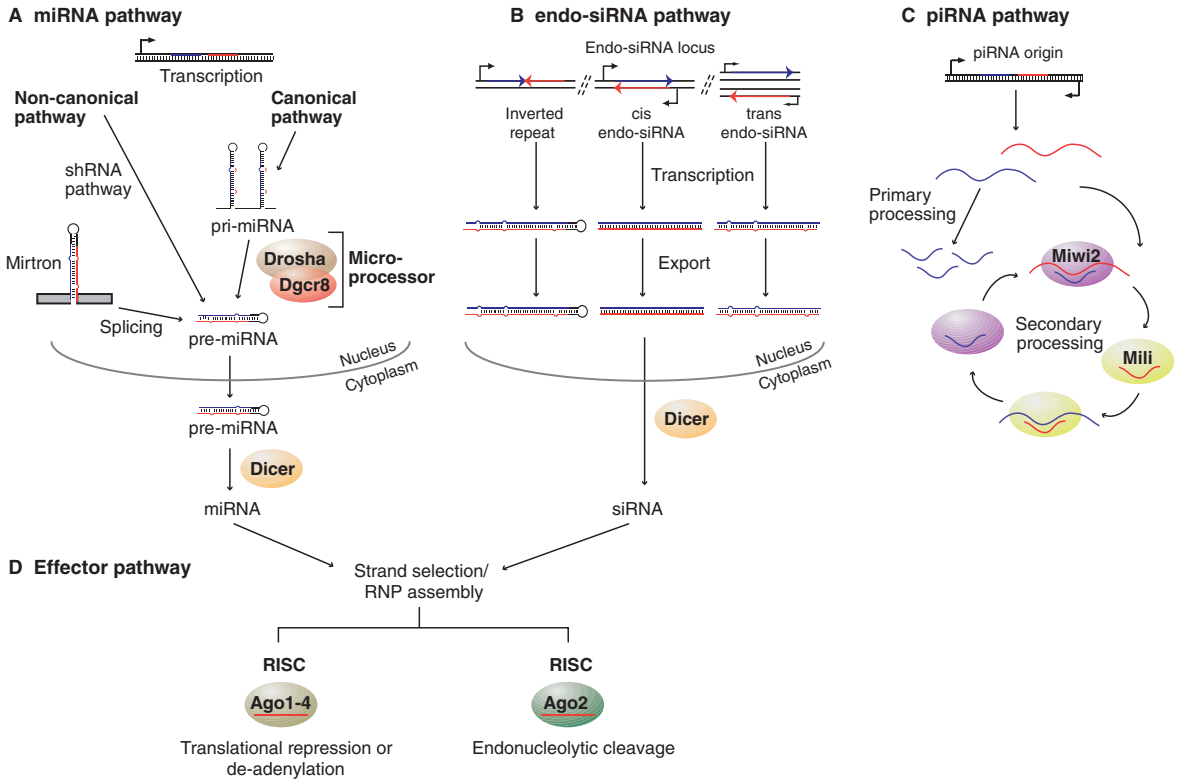


Fig. 1. Biogenesis of microRNAs (miRNAs), endogenous small interfering RNAs (endo-siRNAs) and Piwi-interacting RNAs (piRNAs). (**A**) Canonical miRNAs are processed from long primary miRNAs (pri-miRNAs) into short hairpin precursor miRNAs (pre-miRNAs) by the microprocessor, a complex consisting of the RNA binding protein Dgcr8 and the RNase III enzyme Drosha. By contrast, non-canonical miRNAs are transcribed directly as short hairpins (shRNAs) or derive from introns that can refold into shRNAs (mirtrons). (**B**) Precursors of endo-siRNAs are derived from long stem-loop structures (inverted repeat), opposing strand transcription (cis endo-siRNAs), or gene-pseudogene pairs (trans endo-siRNAs). Both miRNAs and endo-siRNAs are then processed by the RNase III enzyme Dicer to produce double-stranded RNAs of ~21 nucleotides. (**C**) piRNAs are processed from single-stranded RNA precursors that are often encoded by intergenic repetitive elements or transposons. The mechanisms that drive piRNA biogenesis are not well understood, although a 'ping-pong mechanism' has been described for a subset of piRNAs. In this model, Mili cleaves the primary piRNA, which is subsequently recognized by Miwi2. Miwi2 cleaves the other strand of the precursor that can then bind to Mili, thus forming a positive amplification loop. (**D**) Following their processing, miRNAs and endo-siRNAs are assembled into ribonucleoprotein (RNP) complexes called RNA-induced silencing complexes (RISCs). The key components of RISCs are proteins of the Argonaute (Ago) family. In mammals, four Ago proteins (Ago 1-4) function in miRNA repression but only Ago2 functions in siRNA repression. The fate of piRNAs is unknown. On the DNA, blue represents the positive strand and red represents the negative strand.

and Mili in mouse; also known as Piwil1, Piwil4 and Piwil2, respectively). piRNAs are generated from long single-stranded RNA precursors that are often encoded by complex and repetitive intergenic sequences. One proposed model for their biogenesis is the 'ping-pong mechanism' (Fig. 1C) (Aravin et al., 2007; Brennecke et al., 2007; Gunawardane et al., 2007). In this model, the Argonaute protein Mili cleaves the primary piRNA to define the 5′ end of piRNAs, which is subsequently recognized by Miwi2. Miwi2 then cleaves the other strand of the precursor, thereby generating a 5′ end of the piRNA that can then bind to Mili, thus forming a positive amplification loop. Many of the details of this model remain to be uncovered. Furthermore, the ping-pong model is likely to explain the biogenesis of only a subset of mammalian piRNAs, those that are derived from repetitive sequences, such as transposons, and that are associated with Miwi2 and Mili in the early stages of spermatogenesis. The mechanism of biogenesis of piRNAs derived from complex intergenic sequences, associated with Miwi and Mili in the later stages of spermatogenesis, is unclear.

Small RNAs in gametes

Most animals, including vertebrates, reproduce sexually and have the ability to form gametes. The two types of gametes, the egg and the sperm, arise from immature germ cells, undergo extensive differentiation and ultimately fuse to create their progeny. One fascinating aspect of this process is that, upon fertilization, the highly specialized sperm and egg unite to produce the zygote, which is totipotent, having the potential to produce all the cells of the body. This switch from a singular function to a totipotent cell involves massive molecular rewiring (Hemberger et al., 2009). Based on recent findings, small RNAs are likely to play a very important role during this transition.

Small RNAs in spermatogenesis

Spermatogenesis is a highly regulated process, during which diploid spermatogonia differentiate into haploid spermatozoa within the seminiferous epithelium of the testis. During the course of differentiation into sperm, numerous mRNAs are regulated post-transcriptionally (Lee et al., 2009). Recent studies using both

genetics and miRNA profiling on different populations of spermatogenetic cells have identified an important role for miRNAs during spermatogenesis (Hayashi et al., 2008; Tang et al., 2007; Yu et al., 2005; Bouhallier et al., 2010; Yan et al., 2009). Deletion of *Dicer*, for example, results in a loss of sperm (Hayashi et al., 2008; Maatouk et al., 2008). This could be attributed to the loss of either miRNAs or endo-siRNAs. However, the deletion of *Argonaute 2* (*Ago2*), a protein that is essential for cleavage of mRNA targets by endo-siRNAs, has no obvious testis phenotype, suggesting that the Dicer phenotype is predominantly an miRNA-based phenotype (Hayashi et al., 2008). Roles for individual miRNAs during spermatogenesis have also been described. For example, Mir122a regulates Tnp2, a testis-specific gene involved in chromatin remodeling during spermatogenesis (Yu et al., 2005). In addition, Mir34c is highly expressed in germ cells and its overexpression enhances spermatogenesis (Bouhallier et al., 2010). Furthermore, Dead end 1 (Dnd1), an RNA-binding protein that is implicated in prevention of miRNA access to cell cycle-related target mRNAs, is essential for fetal male germ cell development (Cook et al., 2010; Kedde et al., 2007). Recently, it has been also shown that Mir18, a member of the Oncomir-1 cluster of miRNAs, directly targets heat shock factor 2 (Hsf2), a transcription factor involved in spermatogenesis (Bjork et al., 2010).

Genetic studies imply that, like miRNAs, piRNAs are also essential for spermatogenesis. In mouse, piRNAs have been separated into two classes based on the timing of their expression, their repetitive versus nonrepetitive nature, and the Piwi proteins with which they are associated (Aravin et al., 2006; Girard et al., 2006; Grivna et al., 2006; Lau et al., 2006; Watanabe et al., 2006). The first class is highly repetitive and is expressed before meiotic pachytene. This class of piRNAs interacts with Mili and Miwi2 (Aravin et al., 2008; Aravin et al., 2007). The second class of piRNAs is nonrepetitive, becomes abundant during the pachytene stage and is associated with Mili and Miwi proteins (Aravin et al., 2008; Aravin et al., 2007; Girard et al., 2006). Consistent with their timing of expression, deletion of Mili and Miwi2 results in early arrest in meiosis I (at the primary spermatocyte stage), whereas deletion of Miwi results in arrest following meiosis II (the round spermatid stage) (Carmell et al., 2007; Deng and Lin, 2002; Kuramochi-Miyagawa et al., 2004).

The repetitive piRNAs are associated with the repression of transposable elements during spermatogenesis (Malone and Hannon, 2009) but exactly how repression is achieved is unclear. For example, it is unclear whether this repression occurs transcriptionally or post-transcriptionally. There is evidence to suggest that repetitive piRNAs promote de novo demethylation of the transposons (Aravin et al., 2008; Kuramochi-Miyagawa et al., 2008); however the mechanism by which these small RNAs can direct the DNA methylation machinery is unclear.

Small RNAs in oogenesis

Mammalian oogenesis is distinct from spermatogenesis (Matova and Cooley, 2001). The oocyte pool is largely fixed by the end of mouse embryogenesis and oocytes are then induced to mature in response to cyclic waves of hormones, including follicle stimulating hormone and luteinizing hormone. In the mouse, each cycle induces a small pool of oocytes to mature before they are released into the fallopian tube. During maturation, the oocytes undergo germinal vesicle breakdown (GVBD), meiosis I and finally meiosis II, which is only completed following fertilization. Deep sequencing of small RNAs in mouse oocytes has uncovered not only miRNAs and piRNAs, but also a large population of endo-siRNAs (Tam et al., 2008; Watanabe et al., 2006; Watanabe et al., 2008).

An essential role for endo-siRNAs in the mouse oocyte has been inferred by comparing the knockout phenotypes of *Dicer* and *Dgcr8* mutant mice. *Dicer* loss in the oocyte results in meiotic arrest with severe spindle and chromosomal segregation defects (Murchison et al., 2007; Suh et al., 2010; Tang et al., 2007). Furthermore, thousands of mRNAs are misregulated. By contrast, *Dgcr8* loss has no phenotype and mRNA levels remain unchanged (Suh et al., 2010). As Dicer processes both miRNAs and endo-siRNAs, whereas Dgcr8 is essential only for miRNA processing, these findings imply that endo-siRNAs, and not miRNAs, underlie the meiotic defect of *Dicer* knockout oocytes. The loss of *Ago2* results in a similar phenotype to that observed in *Dicer* knockouts, further supporting the role of endo-siRNAs in regulating meiosis in oocytes (Kaneda et al., 2009). The mechanism of action of endo-siRNAs in oocytes is unclear. In *Schizosaccharomyces pombe*, endo-siRNAs are crucial for heterochromatin formation in repeat regions of the genome (Moazed, 2009). However, no such function has been shown convincingly in mammals. It seems more likely that endo-siRNAs in mammals are acting post-transcriptionally (Tam et al., 2008).

It was surprising to find that in the absence of *Dgcr8* in mouse oocytes, mRNA levels are unchanged. miRNAs are present in oocytes, as determined by both deep sequencing and multiplex quantitative PCR-based profiling (Murchison et al., 2007; Tam et al., 2008; Tang et al., 2007; Watanabe et al., 2006; Watanabe et al., 2008). For example, Let-7, Mir22, Mir16-1 and Mir29 are all highly expressed in the oocyte. miRNA profiling following *Dgcr8* deletion confirmed that these and all other miRNAs tested were indeed lost in knockout oocytes (Suh et al., 2010). Furthermore, mRNA profiling and bioinformatic analyses showed that many targets for the expressed miRNAs are present in the oocyte. Therefore, everything is in place for miRNA-based destabilization to occur, but mRNA levels remain unchanged (Suh et al., 2010). Consistent with these findings, reporter assays show robust siRNA activity in mature oocytes, but little to no miRNA function (Ma et al., 2010). Even with artificial 3′ untranslated regions (3′ UTRs) carrying multiple target sites, and the introduction of supraphysiological doses of miRNAs, little suppression in terms of mRNA stability or translation was seen (Ma et al., 2010). Together, these surprising results show that miRNA function is suppressed in fully grown oocytes even though miRNA biogenesis is unaffected and miRNA targets are present. Although the mechanism of suppression is unknown, one hint comes from the finding that P-bodies (processing bodies, see Box 1), in which miRNA destabilization normally occurs (Parker and Sheth, 2007), are lost in maturing oocytes and only reform at the blastocyst stage (Flemr et al., 2010; Swetloff et al., 2009). Whether the loss of P-bodies is a primary or secondary consequence of miRNA functional loss is unclear.

piRNAs are also expressed in mouse oocytes (Watanabe et al., 2008), but the deletion of the Piwi proteins do not produce an oocyte phenotype (Carmell et al., 2007; Deng and Lin, 2002; Kuramochi-Miyagawa et al., 2004). Therefore, it is unclear whether they play any role during oogenesis.

Small RNAs in early embryogenesis

Small RNA function during pre-implantation development

Zygotic deletion of *Dgcr8* or *Dicer* in mice leads to embryonic arrest shortly after implantation between embryonic day (E) 6.5 and E7.5 (Bernstein et al., 2003; Morita et al., 2007; Wang et al., 2007). However, development to E3.5, the blastocyst stage, occurs

Box 1. P-bodies

P-bodies (processing bodies) are discrete cytoplasmic foci that contain proteins involved in mRNA degradation. They are found in eukaryotic cells as well as in somatic cells in plants and yeast (Bashkirov et al., 1997; Cougot et al., 2004; Sheth and Parker, 2003; Xu et al., 2006). P-body proteins are required for diverse post-transcriptional processes: mRNA decay, translational repression, nonsense-mediated mRNA decay and RNAi-mediated repression. In particular, all four Ago proteins (Eystathioy et al., 2003; Liu et al., 2005; Sen and Blau, 2005), GW182 (Eystathioy et al., 2003) and two RNA helicases RCK/p54 (Chu and Rana, 2006) and MOV10 (Meister et al., 2005) have been found in P-bodies, suggesting that miRNA suppression is localized to the P-body. However, it has been proposed that P-body formation is a consequence rather than the cause of miRNA-mediated gene silencing (Eulalio et al., 2007), as when siRNA or miRNA silencing pathways are blocked, P-bodies are not formed (Eulalio et al., 2007). Interestingly, it has been shown that P-body foci are dynamic, increasing or decreasing in size and number depending on the global state of RNA turnover in yeast (Sheth and Parker, 2003). Indeed, recent studies in mouse oocytes and of early mouse embryonic development demonstrated that these foci are regulated developmentally (Flemr et al., 2010; Swetloff et al., 2009) In particular, it was shown that P-bodies are lost in fully grown oocytes and during pre-implantation development (Flemr et al., 2010; Swetloff et al., 2009). It will thus be interesting to know how and when P-bodies are lost or re-stored during early development.

normally. Indeed, maternal and zygotic loss of *Dgcr8* leads to no discernable phenotype in E3.5 embryos (Suh et al., 2010). Therefore, miRNA function must become essential sometime between E3.5 and E7.5. Recently, careful characterization of the zygotic *Dicer* knockout phenotype suggested that an epiblast forms and there is an initiation of gastrulation with expression of the early mesoderm maker brachyury in the posterior epiblast (Spruce et al., 2010). However, the primitive streak fails to elongate and there is a loss of expression of the definitive endoderm markers Hex (Hhex – Mouse Genome Informatics) and Cerl1 (Cer1). A major caveat of these findings is that it is unclear whether all miRNAs were lost in the *Dicer* knockout embryos. Indeed, in situ hybridization suggested equal levels of miRNAs from the Mir290 cluster in *Dicer* versus wild-type embryos. Expression of the Mir290 cluster is initiated with zygotic gene activation (Tang et al., 2007). Hence, their presence in the zygotic *Dicer* knockout blastocyst suggests the perdurance of maternal *Dicer* rather than the miRNAs themselves. Because of this caveat, the earliest roles of miRNAs in mouse development remain unknown. However, the strong proliferation defects seen in *Dicer* and *Dgcr8* knockout mouse embryonic stem (ES) cells (Kanellopoulou et al., 2005; Murchison et al., 2005; Wang et al., 2008; Wang et al., 2007), suggests that miRNAs are likely to play an important role in the expansion of the epiblast. Interestingly, the loss of maternal miRNAs alone results in a decrease in the average number of progeny produced following fertilization by wild-type males (Suh et al., 2010). Together with a lack of pre-implantation phenotype, this finding suggests a role for maternally contributed miRNAs during the peri- and/or post-implantation stages of development, multiple days after fertilization.

The lack of phenotypes following miRNA removal in early mammalian development parallels findings observed in zebrafish: the loss of both maternal and zygotic miRNAs in zebrafish first manifests phenotypes relatively late in development (Giraldez et al., 2005). Indeed, following maternal-zygotic knockdown of Dicer, zebrafish gastrulate and only begin to manifest clear morphogenetic defects during organogenesis. Zygotic loss alone allows organogenesis to proceed normally (Wienholds et al., 2003). Although the phenotypes are more severe in mouse, with zygotic deletion of *Dicer* resulting in arrest prior to gastrulation (Bernstein et al., 2003; Morita et al., 2007; Wang et al., 2007), these studies show how early development can proceed normally in the absence of miRNAs. A striking difference between the mouse and zebrafish phenotypes is the lack of Dicer requirement in the maturing fish oocyte. It will be important to determine whether this difference is secondary to a lack of endo-siRNA function in the zebrafish egg.

An interesting possibility is that the loss of miRNA function during pre-implantation development is a key component of the dramatic reprogramming that occurs during this stage (Hemberger et al., 2009). Indeed, recent profiling experiments suggest a shift from a dominant presence of endo-siRNAs and piRNAs in the oocyte to an miRNA majority as pre-implantation development proceeds (Fig. 2) (Ohnishi et al., 2010). The transition occurs with zygotic gene activation, which follows resetting of the epigenome. This resetting occurs in a remarkably short window of time, between fertilization and E2.5 in mice and slightly later in humans (de Vries et al., 2008). Therefore, it is tempting to speculate that the suppression of miRNA function enables this massive epigenomic reprogramming in preparation for new gene expression.

miRNAs and siRNAs use distinct silencing machinery

A surprising conclusion arising from the comparison of the *Dicer* and *Dgcr8* knockout phenotypes in mouse oocytes along with the miRNA versus siRNA reporter assays is that the effector pathways

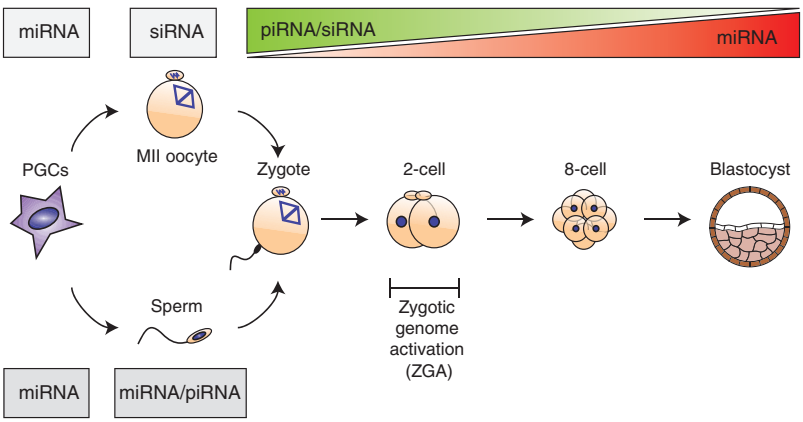
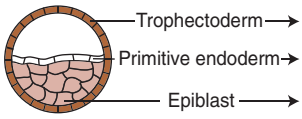


Fig. 2. Small RNA functions during germ cell and early embryonic development. Piwi-interacting RNAs (piRNAs) and microRNAs (miRNAs) are essential in the developing male germline, whereas endogenous small interfering RNAs (endo-siRNAs) play their most crucial role in oocyte maturation. There is a transition from endo-siRNAs or piRNAs to miRNAs during pre-implantation development. PGCs, primordial germ cells.



Stem cells	Gene deleted	Phenotype	Reference(s)
TS cells	*Dicer*	Proliferation defects	Spruce et al., 2010
XEN cells	*Dicer*	Proliferation defects	Spruce et al., 2010
ES cells	*Dicer* or *Dgcr8*	Proliferation and differentiation defects	Kanellopoulou et al., 2005; Murchison et al., 2005; Wang et al., 2007

Fig. 3. Small RNA functions in stem cells derived from the mouse blastocyst. By the time of implantation, the mammalian blastocyst has developed three different cell lineages: trophectoderm, primitive endoderm and epiblast (shown on left). Three distinct self-renewing cell lines can be derived from these lineages: trophoblast stem (TS) cells, extra-embryonic endoderm (XEN) cells and embryonic stem (ES) cells. Studies of stem cell lines lacking either *Dgcr8* or *Dicer* provide insights into small RNA-mediated regulation of stem cell maintenance, proliferation and differentiation (right).

for miRNA and endo-siRNA activity are separable (Fig. 1). That is, although miRNA-based destabilization (by translational inhibition) function is lost, siRNA cleavage function remains. In *Drosophila*, Ago1, together with Loquacious, is the primary driver of miRNA function, whereas Ago2, together with its partner R2D2, is primarily responsible for siRNA function (Ghildiyal and Zamore, 2009). Mammals, by contrast, have four Argonaute proteins: Ago1-4 (Siomi and Siomi, 2009). Ago2 is the only mammalian Argonaute with slicer activity and hence the only Argonaute protein able to perform siRNA cleavage. Indeed, deletion of *Ago2* in oocytes produces a phenotype very similar to that of *Dicer* (Kaneda et al., 2009). However, the Argonautes are highly redundant in terms of miRNA activity. Although deletion of all four Argonautes in ES cells results in complete loss of miRNA function, re-introduction of any one of the four Argonautes can fully rescue miRNA activity (Su et al., 2009). Therefore, unlike the situation in *Drosophila*, siRNA and miRNA function in mammalian cells is unlikely to be compartmentalized at the level of the Argonaute proteins. Instead, proteins associated with or mechanisms downstream of Argonautes must be influencing the specific loss of miRNA function. It is unclear what these mechanisms might be. P-bodies are lost concurrently with miRNA functional loss (Flemr et al., 2010; Swetloff et al., 2009); therefore, studies of the components of the P-body might provide hints.

miRNA versus endo-siRNA activity in other tissues

Microprocessor components have also been knocked out in other tissues and the resulting phenotypes compared with corresponding *Dicer* phenotypes. In particular, *Dgcr8* has been knocked out in skin and cardiomyocytes (Rao et al., 2009; Yi et al., 2009), and *Drosha* has been knocked out in T cells (Chong et al., 2008). In these cases, the microprocessor-null phenotypes were very similar to those of the corresponding *Dicer*-null phenotypes. An exception appears to be in the adult brain, where the deletion of *Dicer* in post-mitotic neurons produces a more severe phenotype than does *Dgcr8* loss (J. E. Babiarz, R. Hsu, C. Melton, E. M. Ullian and R.B., unpublished). However, deep sequencing failed to uncover any evidence of endo-siRNAs in the brain (J. E. Babiarz, R. Hsu, C. Melton, E. M. Ullian and R.B., unpublished). Instead, many non-canonical miRNAs were found, suggesting that these small RNAs might underlie the differences observed. These results suggest that endo-siRNAs might be specific to oocytes and early embryonic development.

Small RNAs in stem cells

Embryonic stem cells

Three self-renewing cell types can be derived from the late mouse blastocyst: ES cells, which represent the pluripotent epiblast lineage; trophoblast stem (TS) cells, which represent the trophoblast lineage; and extra-embryonic endoderm (XEN) cells, which represent the primitive endoderm lineage (Fig. 3) (Rossant, 2008). Insights into small RNA regulation of stem cell maintenance and differentiation have been gained mostly from studies of ES cells lacking either *Dgcr8* or *Dicer* (Kanellopoulou et al., 2005; Murchison et al., 2005; Wang et al., 2007). Both *Dgcr8*- and *Dicer*-deficient ES cells exhibit proliferation and differentiation defects. The proliferation phenotype is associated with accumulation of cells in the G1 phase of the cell cycle, whereas the differentiation defect is associated with an inability to silence the self-renewal machinery (Wang et al., 2007). By adding back individual miRNAs into *Dgcr8* knockout ES cells, a large family of miRNAs, including members of the Mir290 and Mir302 clusters, were found to rescue the prolonged G1 phenotype (Wang et al., 2008). These miRNAs were termed the ESCC miRNAs (ESC cell cycle regulating miRNAs). Using a similar add-back strategy, the Let-7 (also known as Mirlet7) miRNA family was shown to rescue the ability to silence self-renewal (Melton et al., 2010). Specifically, the addition of Let-7 into *Dgcr8* knockout cells led to the loss of expression of multiple markers of ES cells and blocked the ability of cells to reform colonies, functionally proving a loss of their self-renewal capacity. However, Let-7 only silenced self-renewal in the *Dgcr8* knockout, not wild-type ES cells, suggesting that miRNAs normally expressed in ES cells suppress the capacity of Let-7 to induce differentiation. Indeed, simultaneous introduction of the ESCC miRNAs into the *Dgcr8* knockout ES cells suppressed the capacity of Let-7 to induce differentiation. Microarray profiling of mRNAs and bioinformatic analyses showed that these two antagonizing miRNA families function by having opposing effects on members of the pluripotency regulatory network, including Myc, Lin28 and Sall4 along with others (Fig. 4) Taken together, these findings show that the two miRNA families, the Let-7 and ESCC miRNAs, play opposing roles in controlling the balance between ES cell self-renewal and differentiation (Melton et al., 2010).

Endo-siRNAs have also been identified in ES cells (Babiarz et al., 2008). Interestingly, there is no overlap between the specific endo-siRNAs expressed in oocytes and those expressed in ES cells, showing that they are developmentally regulated and likely to have distinct functions (Babiarz et al., 2008). However, the role of ES cell endo-siRNAs is unclear. A hint comes once again from the deletion of members of the biogenesis and effector pathways. Deletions of *Dicer* or of all four Argonaute genes have more severe phenotypes than deletion of *Dgcr8* (Kanellopoulou et al., 2005; Murchison et al., 2005; Su et al., 2009; Wang et al., 2007). In particular pan-Argonaute-deficient ES cells undergo apoptosis. *Dicer*-null ES cells can survive, but during their derivation they go through a phase of arrest, with escaper cells eventually proliferating. By contrast, *Dgcr8*-null ES cells survive and show

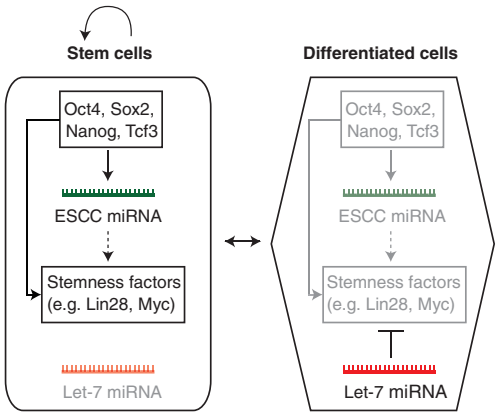


Fig. 4. The opposing roles of ESCC and Let-7 microRNAs (miRNAs) in the switch between self-renewal and differentiation. In mouse ES cells (left panel), ESCC miRNAs (green) and stemness factors are highly expressed. ESCC miRNAs are regulated by the core ES cell transcription factors such as Oct4, Sox2, Nanog, Tcf3 and Myc. Upon differentiation (right panel), the expression of Let-7 miRNAs (red) increases and helps to repress stemness factors such as Lin28, Myc and Sall4.

no evidence of arrest during derivation. These differences in phenotype suggest a likely role for endo-siRNAs in ES cells. By contrast, a role for piRNAs in the embryonic stem cells is doubtful as their levels are greatly diminished relative to those observed in the germline (Ohnishi et al., 2010), and the knockout of the Piwi genes in mice show no embryonic phenotypes (Carmell et al., 2007; Deng and Lin, 2002; Kuramochi-Miyagawa et al., 2004).

Trophoblast stem cells

During the course of early embryogenesis, the separation of the trophectoderm and ICM lineages is the first known definitive differentiation event. Fundamental insights into the molecular control of trophectoderm determination and differentiation have been made in the past decade (Chen et al., 2010; Douglas et al., 2009; Ralston and Rossant, 2005). A number of transcription factors and signaling pathways have been identified as crucial players. Even before the formation of the blastocyst, two transcription factors, Oct4 (Pou5f1 – Mouse Genome Informatics) and Cdx2, act antagonistically to establish the boundaries between the trophectoderm and inner cell mass (Niwa et al., 2005). Oct4 is expressed throughout pre-implantation development whereas Cdx2 is expressed starting around the time of morula compaction (Dietrich and Hiiragi, 2007). At first, Cdx2 is co-expressed with Oct4 in the cells of the morula, but its expression is then seen to segregate to the future trophectoderm cells. Cdx2 protein binds directly to Oct4 protein resulting in reciprocal inhibition of their target genes (Niwa et al., 2005). Dominance of one protein over the other eventually leads to the choice between the two lineages: inner cell mass versus trophectoderm. A second transcription factor, Eomes, which acts independently of Cdx2, is also essential early in trophectoderm determination and maintenance, but little more is known about its function (Russ et al., 2000). Following the formation of the blastocyst, the inner cell mass produces fibroblast growth factor 4 (Fgf4), which signals through the FGF receptor 2 (Fgfr2), to promote proliferation of the overlying polar trophectoderm, thereby allowing the polar trophectoderm to provide an ongoing source of trophoblasts both to the mural trophectoderm and future placenta (Nichols et al., 1998; Tanaka et al., 1998).

In contrast to the ES cell studies, little is known about the role of small RNAs in trophectoderm specification. miRNA expression profiling of ES cells, ES cell-derived TS cells and progressive stages of pre-implantation embryos, has identified a subset of miRNAs that might play a role in trophectoderm specification: Mir297, Mir96, Mir21, Mir29c, Let-7, Mir214, Mir125a, and Mir424 (Viswanathan et al., 2009) Moreover, studies analyzing the phenotype of *Dicer*-deficient embryos during early post-implantation stages have shown an essential role for small RNAs in trophectoderm development (Spruce et al., 2010). In particular, expression of the TS cell markers *Eomes*, *Cdx2* and *Esrrb* was greatly downregulated in *Dicer* knockout embryos. Similar to ES cells, *Dicer* knockout TS cells show proliferation defects, with an accumulation of cells in G1. This finding is consistent with the fact that the Mir290 cluster is also highly expressed in TS cells (Houbaviy et al., 2005). Indeed, as seen in ES cells, a number of inhibitors of the cyclin E/Cdk2 pathways were upregulated in TS cells following miRNA loss (Spruce et al., 2010). *Dicer* removal in XEN cells also influenced self-renewal and proliferation, but potentially through different pathways. In particular, regulation of ERK activity appears to be an important player in the phenotype. Taken together, early experiments in TS and XEN cells suggest overlap in miRNA roles across the three stem cell populations of the embryo.

Induced pluripotent stem cells

In 2006, Yamanaka and co-workers showed that somatic cells could be reprogrammed into induced pluripotent stem (iPS) cells by retroviral introduction of genes encoding four transcription factors: Oct3/4 (Pou5F1 – Mouse Genome Informatics), Klf4, Sox2 and Myc (Takahashi and Yamanaka, 2006). With improvements in the methods, these iPS cells have become increasingly similar to ES cells both in their self-renewal and differentiation potential (for a review, see Amabile and Meissner, 2009). The realisation of the importance and therapeutic potential of iPS cells has opened a new era in regenerative medicine (Yamanaka, 2009).

A role for miRNAs in iPS cell production has recently been uncovered. In particular, ESCC miRNAs can promote the de-differentiation of somatic cells to iPS cells. They can replace Myc and, based on chromatin immunoprecipitation (ChIP) sequence data, function downstream of Myc (Judson et al., 2009). Interestingly, ESCC miRNAs also upregulate Myc, albeit indirectly (Melton et al., 2010). Therefore, ESCC miRNAs and Myc form a self-reinforcing loop that maintains ES cell self-renewal and even promotes de-differentiation. Furthermore, inhibition of Let-7 function, either through overexpression of Lin28, which blocks Let-7 biogenesis, or through antagomirs, which directly target mature Let-7, is able to promote iPS cell production (Melton et al., 2010; Yu et al., 2007). This result is consistent with Let-7's capacity to suppress Myc and many of the downstream targets of the pluripotency network of transcription factors. These findings further emphasize the role of these miRNAs in regulating the switch between self-renewal and differentiation.

Emerging mechanisms of miRNA regulation

In the past decade, much progress has been made in identifying miRNAs, understanding miRNA biogenesis and predicting miRNA targets. Furthermore, it is becoming evident that miRNAs can exert their effects through single or multiple targets, allowing them to regulate development, normal physiology, and pathological processes. However, there remains much to be learned about miRNA biology. A question of increasing interest is

how miRNAs themselves are regulated. However, the answers to this question are almost as diverse as the miRNAs identified and, therefore, below we discuss only those mechanisms that regulate small RNAs with known roles in germ cells and in the gastrulating embryo [for a broader review on the topic, see Krol et al. (Krol et al., 2010)].

Most miRNAs are expressed through an RNA polymerase II mechanism, and regulation of their expression thus occurs through the common mechanisms that regulate developmental genes. For example, the expression of ESCC miRNAs is regulated by the core ES cell transcription factors Oct4, Sox2, Nanog, Tcf3 and Myc (Judson et al., 2009; Marson et al., 2008). Furthermore, and as observed for other pluripotency genes, the expression of ESCC miRNAs is regulated by epigenetic modifications, which include activating and suppressive histone marks (Judson et al., 2009; Marson et al., 2008).

miRNAs are also regulated post-transcriptionally at the level of their biogenesis and stability. For example, Lin28, an RNA binding protein, regulates the biogenesis of the Let-7 family of miRNAs (Heo et al., 2008; Newman et al., 2008; Piskounova et al., 2008; Rybak et al., 2008; Viswanathan et al., 2008). Lin28 inhibits Dicer cleavage and destabilizes the pre-miRNA form of Let-7 (Heo et al., 2008; Rybak et al., 2008). The latter is achieved through Lin28 interacting with the loop region of Let-7 and directing a terminal uridyl transferase (TUTase) to polyuridylate the 3′ end of the pre-Let-7 miRNA, leading to pre-Let-7 degradation (Hagan et al., 2009; Heo et al., 2009).

miRNA function can also be regulated by interactions with their downstream targets. For example, the RNA binding protein dead end (DND1) blocks the ability of MIR221 and MIR372 to suppress p27 (CDKN1B – Human Gene Nomenclature Database) and LATS2, respectively, in human cells (Kedde et al., 2007). DND1 is essential for germline development in zebrafish and mouse, where it probably plays a similar role to that seen in the human cell lines (Kedde et al., 2007; Slanchev et al., 2009). Another germline RNA binding protein, Dazl (deleted in azoospermia-like), has also been shown to have a potential role in regulating the interactions between miRNAs and mRNA targets in zebrafish (Takeda et al., 2009). In particular, Dazl appears to inhibit miRNA induced de-adenylation by binding to the 3′UTRs of specific miRNA targets.

Future directions

Over the past decade we have learned a great deal about the nature of small RNAs, their biogenesis and their expression across tissues. However, we are just beginning to appreciate how these RNAs fit in to the overall molecular network of the cell. Indeed, we have a very poor understanding of how the many mRNA targets of individual miRNAs work together to influence a specific cellular outcome. To date, the field has mostly limited the analysis of miRNA targeting in biological processes to a small number of targets, probably not reflecting the true nature of miRNA function. Future studies involving more systematic approaches should teach us about how miRNAs are involved in the control of development, tissue physiology and disease.

Furthermore, there remains much to be learned about the transcriptional, epigenetic and post-transcriptional regulation of miRNAs. miRNA targets are likely to be heavily influenced by cellular context. The influence of cellular context can, in part, be explained by differences in expression of those targets. However, a large part will probably be attributable to the regulation of miRNA biogenesis and the interaction of miRNAs with their target mRNAs. The most extreme example is the global suppression of miRNAs in the oocyte. The reason and mechanistic details of this global suppression remain unclear. How the global or focused regulation of miRNA maturation and targeting fit in with the timing and regulation of cell fate decisions and their conservation across species should be a fruitful area of research. Finally, it will be important to study the relationship of these regulatory mechanisms to the biology and treatment of disease, including within the field of cellular reprogramming.

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Competing interests statement

The authors declare no competing financial interests.

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