CHAPTER SIX

Small RNAs in Germline Development

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Abstract

One of the most important and evolutionarily conserved strategies to control gene expression in higher metazoa is posttranscriptional regulation via small regulatory RNAs such as microRNAs (miRNAs), endogenous small interfering RNAs (endo-siRNAs), and piwi-interacting RNAs (piRNAs). Primordial germ cells, which are defined by their totipotent potential and noted for their dependence on posttranscriptional regulation by RNA-binding proteins, rely on these small regulatory RNAs for virtually every aspect of their development, including specification, migration, and differentiation into competent gametes. Here, we review current knowledge of the roles miRNAs, endo-siRNAs, and piRNAs play at all stages of germline development in various organisms, focusing on studies in the mouse.

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1. INTRODUCTION: GERMLINE DEVELOPMENT AND SMALL REGULATORY RNAs

Germ cells represent the immortal line: they are guardians of a totipotent genome and are essential for the genetic perpetuation of an individual organism and, ultimately, a species. An error at any stage in development (specification, migration, colonization, differentiation, adult maintenance) can lead to disastrous outcomes, including germ cell death or unchecked proliferation leading to tumorigenesis. Tight regulation of germ cell development occurs at both genetic and epigenetic levels and is essential to prevent the untimely manifestation of latent totipotency at any stage other than fertilization when the gametes fuse to complete the cycle of reproduction.

Small RNAs have proven to be robust regulators of pluripotency and cell fate in many contexts, including germ cells. Three major classes of small regulatory RNAs have been discovered: microRNAs (miRNAs), endogenous small interfering RNAs (endo-siRNAs), and piwi-interacting RNAs (piRNAs). Though each small RNA pathway is functionally distinct, related proteins are required for small RNA biogenesis and function in all pathways. All three types of small RNAs are present in germ cells and are required for critical events in germline development including primordial germ cell (PGC) specification, migration, gonad colonization, sex-specific differentiation into competent gametes, and fertilization, resulting in the early development and specification of the next founding PGC population. Using examples from vertebrate and invertebrate species, this chapter will review the origin and function of each type of small RNA during germline development, from PGC to gamete and back again.

1.1. Germ cell life cycle and posttranscriptional regulation of gene expression

Germ cell specification, development, and differentiation involve complex regulatory networks at the transcriptional as well as translational levels. In many species, germ cells contain a cytoplasmic RNA-rich regulatory center known generally as a germ granule, indicating a central role for translational regulation in the germline (Seydoux & Braun, 2006). In fact, at particular stages in germ cell development, transient transcriptional quiescence highlights the importance of posttranscriptional regulatory mechanisms involving small RNAs and RNA-binding proteins (RBPs). Regulation of translation involves choreographed interactions of the messenger RNA

(mRNA) with both RBPs and various classes of small regulatory RNAs, including miRNAs, endo-siRNAs, and piRNAs.

The miRNAs function by destabilizing and suppressing the translation of target transcripts. Targeting occurs through partial complementation between the miRNA and the target mRNA. The mechanism by which endo-siRNAs and piRNAs function is less clear, but interactions with complementary RNAs or chromatin-modifying complexes are likely to be involved. RBPs can either promote or antagonize small RNA activity, depending on the molecular milieu and cellular context (Kedde & Agami, 2008). In this chapter, we will review the role of small RNAs at different stages of germ cell development, focusing largely on work reported from mice, but also including related mechanisms in other major genetic model organisms such as the fly, nematode, and zebrafish. The following sections will provide a very brief overview of mouse germline development. Figure 6.1 summarizes the life cycle of germ cells in the mouse and provides a timeline of major events. For a more comprehensive review of the different stages of germ cell development in mouse and other metazoa, please see the following references: for PGC specification (Durcova-Hills & Capel, 2008; Raz, 2003; Saitou & Yamaji, 2010; Santos & Lehmann, 2004), PGC migration (Kimble & Crittenden, 2005; Kunwar, Siekhaus, & Lehmann, 2006; Tarbashevich & Raz, 2010), and PGC gonad colonization and differentiation (Fuller & Spradling, 2007; Kimble, 2011; Western, 2009).

1.1.1 PGC specification and migration

An interesting feature of animal germ cells is that they are specified very early in development, long before the formation of the gonad, which is the site where germ cells will later reside and carry out their function of gametogenesis (Fig. 6.1A). This means that PGCs are molecularly distinguished from their somatic neighbors at a very early stage in the embryo and must then migrate through developing tissues to reach the nascent gonad, maintaining a germ cell identity as they transit through a multitude of somatic cell types and associated molecular signals.

Depending on the species, PGCs are specified through a process of either "preformation" or "induction." Invertebrate species such as *Drosophila melanogaster* (fly) and *Caenorhabditis elegans* (nematode), as well as vertebrate species like *Danio rerio* (zebrafish) and *Xenopus laevis* (frog) utilize "preformation," or the inheritance of maternally deposited germ plasm components, to specify PGCs (Strome & Lehmann, 2007). In other words, RNAs and/or

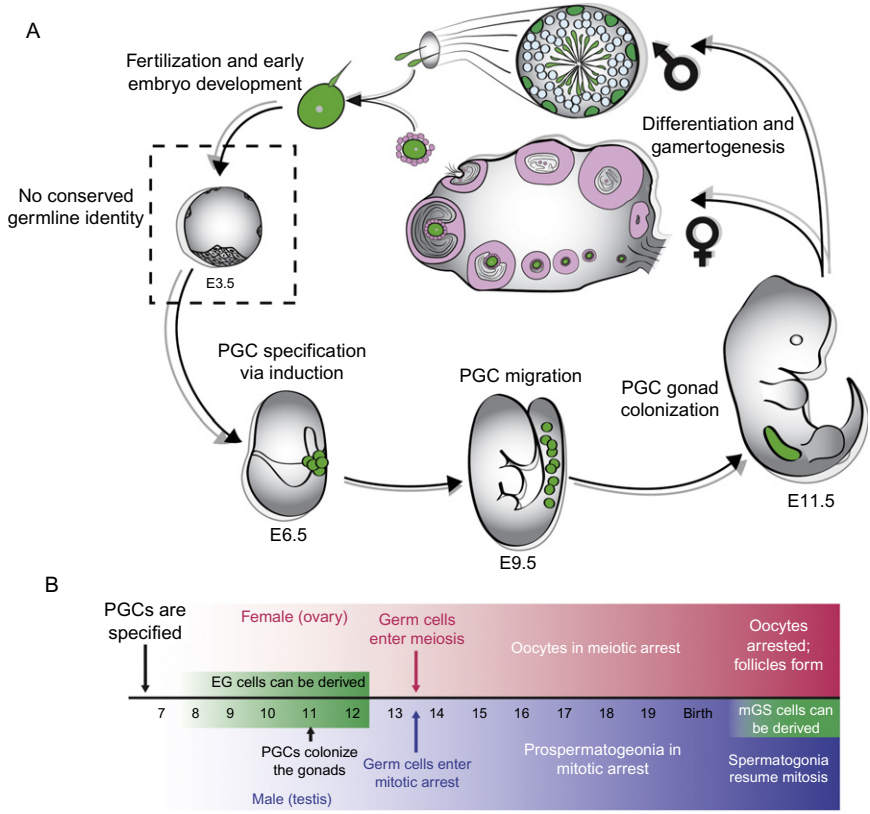

Figure 6.1 The life cycle of a mouse germ cell and a timeline of events. (A) Primordial germ cells (PGCs) are specified (green) from somatic progenitors in the developing mouse embryo around embryonic day 6.5 (E6.5). At this point, there is no gonad organ to populate and begin gametogenesis. Instead, PGCs migrate through the developing hindgut (E9.5) and enter the genital ridges to colonize the gonad by E11.5. After sex-specific differentiation into either mature sperm or an egg, haploid gametes fuse during fertilization and generate a totipotent zygote that gives rise to a blastocyst at E3.5. The dashed box indicates the stage during mouse development where no recognizable germline cell or conserved germ plasm component is expressed. This feature distinguishes the mouse from other vertebrate (zebrafish and frog) and invertebrate (fly and nematode) models, where the germ cell lineage is continuous and PGCs are induced via inheritance of germ plasm components maternally deposited in the mature oocyte. (B) A timeline of important events in the life cycle of mouse germ cells. PGCs are specified around E6.5. During the time that PGCs migrate and colonize the gonads, they can be explanted to form embryonic germ cells (EGCs), which resemble embryonic stem cells (ESCs) in many aspects. After gonad colonization, PGCs commit to their sex-specific fate: prospermatogonia in the male and oogonia in the female. Male germ cells enter mitotic arrest, and female germ cells immediately enter meiosis. After birth, male germ cells resume mitosis and maintain a spermatogonial stem cell that can be cultured to form multipotent germline stem (mGS) cells. Female germ cells associate with somatic granulosa cells to form follicles that will be induced to mature by hormones.

proteins that define the germ cell fate are localized to a region of the early embryo such that the cells forming around those components "inherit" those properties and adopt the germ cell fate. Alternatively, in mammalian species, most notably the mouse (*Mus musculus*), specific signaling molecules instruct a small population of cells to adopt a PGC fate in a process known as "induction." Most evidence suggests that BMP4 induces mouse PGCs to form from somatic progenitors of the epiblast at embryonic day 6.5 (E6.5) amidst larger patterning events along the border of the extraembryonic ectoderm (Lawson et al., 1999; Ohinata et al., 2009; Tam & Zhou, 1996). The lack of expression of conserved germline genes in any cells of the early embryo preceding the induction of PGCs (Fig. 6.1A, dashed box) is a marked difference from species whose "preformation" strategy ensures an unbroken cellular lineage with the germline fate. In mouse, this method of induction is consistent with the highly regulative nature of early mammalian development. For example, early cell divisions appear to be symmetrical, with each cell maintaining equal potency to make all cell types. Distinct cell fates are only determined once inside–outside asymmetry occurs, just prior to the formation of the inner cell mass and trophectoderm at E2.5; PGCs are induced following establishment of anterior–posterior asymmetry 4 days later (E6.5).

The molecular basis of PGC specification in mice (Fig. 6.1A, E6.5) involves the silencing of genes associated with somatic cell differentiation (e.g., Hox genes). Suppression of somatic differentiation occurs through the action of the transcription factor BLIMP1 and associated chromatin-modifying complexes (Ancelin et al., 2006; Saitou, 2009), maintenance of the pluripotency transcriptional network (*Oct4*, *Nanog*, *Sox2,* and *Stella*), and activation of germ cell-specific genes (e.g., *Dnd1*, *Tdrd1*, and *Nanos3)*, many of which are RBPs involved in translational regulation (Ohinata et al., 2006; Saitou, Barton, & Surani, 2002; Yabuta, Kurimoto, Ohinata, Seki, & Saitou, 2006).

In *Drosophila*, PGCs form via preformation at the posterior pole where they inherit the germ plasm. Genes required for specifying PGC fate include *oskar*, *nanos*, *germ cell-less* , and *polar granule component*. Oskar is critical for defining the posterior region and localizing germ plasm components (Lehmann & Nusslein-Volhard, 1991), while Pgc inhibits phosphorylation of RNA polymerase II, thus suppressing transcription (Strome & Lehmann, 2007). The evolutionarily conserved RBP Nanos block somatic differentiation and is critical for germ cell specification and maintenance in many species from planaria to mouse (Forbes & Lehmann, 1998; Koprunner, Thisse,

Thisse, & Raz, 2001; Lehmann & Nusslein-Volhard, 1991; Subramaniam, 1999; Suzuki, Tsuda, & Saga, 2007; Tsuda et al., 2003; Wang, Zayas, Guo, & Newmark, 2007). A gradient of *nanos* in the fly embryo is necessary for posterior patterning and germ cell specification; this gene acts as a translational repressor of *hunchback* as well as other target transcripts (Irish, Lehmann, & Akam, 1989). These coordinated events restrict germ plasm transcripts to the pole cells of the fly embryo, thus specifying the PGC fate.

Once specified, PGCs in all species migrate to the site of the developing gonad via passive and active mechanisms. In *Drosophila*, migration is initially passive as the blastoderm folds and PGCs transition to the posterior midgut (Santos & Lehmann, 2004). The situation is similar in zebrafish and mouse where initial tissue growth and reorganization repositions the founding PGCs as they become motile. Whereas fly and mouse PGCs develop as one founding population, there are four founding populations of PGCs in zebrafish (totaling about 40–45 germ cells) that eventually make their way to the gonads (Raz, 2003; Weidinger, Wolke, Koprunner, Klinger, & Raz, 1999; Weidinger et al., 2002). Although the specification pattern of the founding PGC population differs in zebrafish, many of the same specification and migration cues are conserved, including Nanos (Koprunner et al., 2001) and Cxcr4 (Knaut, Werz, Geisler, & Nusslein-Volhard, 2003), respectively.

Mouse PGCs enter the hindgut and migrate along the dorsal aorta (Fig. 6.1A, E9.5) until they reach the genital ridge. During this time, they proliferate approximately every 16 h (Buehr, McLaren, Bartley, & Darling, 1993) and require specific survival factors to prevent apoptosis and promote cell division. Several factors known to be required for survival *in vivo* include *c-kit*, *kit-l*, TIAR, *Fgfr2-IIIb*, *Nanos3*, *Dnd1*, *Mvh*, *Ror2*, and *Wnt5a* (Beck, Miller, Anderson, & Streuli, 1998; Laird, Altshuler-Keylin, Kissner, Zhou, & Anderson, 2011; Sakurai, Iguchi, Moriwaki, & Noguchi, 1995; Suzuki, Tsuda, Kiso, & Saga, 2008; Takeuchi, Molyneaux, Runyan, Schaible, & Wylie, 2005; Tanaka et al., 2000; Zhao & Garbers, 2002). During active migration, guidance cues such as SDF-1 and its receptor CXCR4 (Molyneaux et al., 2003) as well as *c-kit* and *kit-l* (Buehr et al., 1993; Mintz & Russell, 1957) are necessary to keep PGCs from traveling off course. PGCs that migrate to ectopic locations are eliminated by BAX-mediated apoptosis (Stallock, Molyneaux, Schaible, Knudson, & Wylie, 2003). From a founding population of only a few dozen PGCs, thousands will reach the genital ridges to populate the nascent gonads (Godin, Wylie, & Heasman, 1990).

1.1.2 Gonad colonization and early differentiation

Once PGCs arrive in the gonad, they begin to commit to their sex-specific fate and are highly regulated by their somatic environment. In *Drosophila*, there are both male and female germline stem cells, and their respective gonadal niches closely regulate their cell cycle and differentiation. Alternatively, in mouse, only males have germline stem cells; females are instead endowed with a limited number of immature oocytes that will mature with each female ovulatory cycle until the original supply is depleted.

Mouse PGCs commit to their sex-specific fate after arriving in the somatic gonad at E11.5 (Fig. 6.1A) and will differentiate into sperm or egg depending on whether they develop in a testis or an ovary. PGCs in a testis will differentiate into prospermatogonia, later giving rise to mature sperm; PGCs in an ovary will differentiate as oogonia, later giving rise to mature eggs. The somatic environment directly controls the differentiation of germ cells into either sperm or eggs. Survival and commitment of germ cells to the male fate is dependent on expression of *Fgf9* in somatic cells (DiNapoli, Batchvarov, & Capel, 2006) and *Nanos2* in germ cells (Suzuki & Saga, 2008). Null mutations of these genes, or other downstream genes involved in blocking the activity of retinoic acid, like *Cyp26b1* (Bowles et al., 2006), lead to disruptions of male development. Commitment of germ cells to the female fate is, in turn, marked by immediate entry into meiosis (McLaren, 1984) and upregulation of genes associated with meiotic recombination (Menke, Koubova, & Page, 2003).

During gonad colonization, major differences arise in the gene expression and cell cycle profiles of male and female PGCs (Fig. 6.1B). Male germ cells enter mitotic arrest in G0 shortly after they reach the gonad and remain arrested until after birth (Matsui, 1998; McLaren, 1984; Western, Miles, van den Bergen, Burton, & Sinclair, 2008). Genes associated with the initiation of mitotic arrest include the cell cycle regulators *p27* (*Cdkn1b*), *p21* (*Cdkn1a*), and *p16* (*Cdkn2a*) (Western et al., 2008). Male germ cells do not resume mitosis until after birth, at which point prospermatogonia move to the periphery of testis cords, resume mitosis, and establish the male germline stem cell population of the adult testis (referred to as spermatogonial stem cells, SSCs). SSCs retain the potential to renew their own population but are normally restricted to give rise only to sperm throughout the lifetime of the male.

In the ovary, retinoic acid signaling and germ cell-specific *Dazl* expression are required for female germ cells to enter meiosis at E13.5, where they arrest at the end of the first prophase (Bowles et al., 2006; Bowles &

Koopman, 2010; Gill, Hu, Lin, & Page, 2011). The oocytes associate with *Foxl2*-expressing granulosa cells (Mork et al., 2012) to form primordial follicles that remain arrested at prophase I until the females reach sexual maturity. Oocyte maturation occurs as a result of signaling by cyclic waves of hormones, including follicle stimulating (FSH) and lutenizing hormone (LH). Once the ovary has been depleted of follicles, there is no female germline stem cell to replenish the pool of competent oocytes for fertilization. This is in stark contrast to females of many other invertebrate and vertebrate species such as fly and zebrafish, where oocytes continue to be produced throughout much of the lifetime of the organism.

1.1.3 Gametogenesis and fertilization

In mammals, male gametogenesis begins in the male gonad with the establishment of a SSC, which can self-renew and/or produce daughter cells that differentiate (de Rooij & Grootegoed, 1998). *In vitro*, these SSCs can be explanted to culture where they form multipotent germline stem (mGS) cells (Kanatsu-Shinohara et al., 2004). *In vivo*, these differentiating spermatogonia give rise to several intermediate cell types before forming type B spermatogonia. Type B cells develop into primary spermatocytes, enter meiosis I to produce secondary spermatocytes, and finally complete meiosis II to form early and late spermatids (Bellve et al., 1977).

Spermatogenesis and oogenesis in mammals share the common thread of meiosis, but each is quite distinct from the other in several aspects (Fig. 6.1A, top; Matova & Cooley, 2001). Whereas male meiosis will produce four gametes from a single meiotic division, only one egg is produced from a female meiotic division. Once females reach sexual maturity, oocytes arrested at prophase I of meiosis are recruited in small numbers by hormones (FSH and LH) to produce mature follicles. Maturing follicles contain oocytes intimately associated with somatic support cells that regulate meiotic reentry, leading ultimately to germinal vesicle breakdown in the oocyte, completion of meiosis I, arrest at metaphase II, and ovulation into the fallopian tube, where they await fertilization (for a review of this process, see Russell & Robker, 2007).

Fertilization, the fusion of mature gametes, is a critical reprogramming event where a terminally differentiated oocyte transitions to a totipotent zygote. The fusion of haploid genomes and reorganization of chromatin at this critical developmental stage is accompanied by transcriptional quiescence, emphasizing the importance of posttranscriptional regulation of gene expression. The storage and localization of maternal transcripts in the mature

oocyte is essential for fertilization and early development in many species. Thus, small RNAs and RBPs are critical at this stage to ensure that normal translation programs facilitate the oocyte-to-zygote transition.

1.2. Small RNAs

The different classes of small regulatory noncoding RNAs include miRNAs, endo-siRNAs, and piRNAs. These small RNAs range in size from 18 to 32 nucleotides (nts), and they differ in both their biogenesis and function. The miRNAs function largely by suppressing translation of target transcripts (Bazzini, Lee, & Giraldez, 2012; Djuranovic, Nahvi, & Green, 2012). While little is known about mammalian endo-siRNA function, their exogenously synthesized counterparts function by cleavage and hence direct destabilization of target mRNA (Montgomery & Fire, 1998; Novina & Sharp, 2004). In fission yeast, endo-siRNAs are involved in the spreading of heterochromatin (Cam et al., 2005; Verdel et al., 2004), though there is little evidence to suggest such a role in mammals. In contrast, there is good evidence that piRNAs function, at least in part, at the transcriptional level via epigenetic modifications to the genome (Kuramochi-Miyagawa et al., 2008).

The burgeoning field of small RNA biology began with the discovery of a genetic mutation in a region of the genome coding for small RNAs that are complementary to the transcript of another gene (Lee, Feinbaum, & Ambros, 1993; Wightman, Ha, & Ruvkun, 1993) and the subsequent evidence that double-stranded RNA (dsRNA) could alter gene expression through a process known as RNA interference (RNAi) (Fire et al., 1998; Montgomery, Xu, & Fire, 1998). Further research has revealed that these small regulatory RNAs exist in large numbers across many species from yeast to plants to animals. The following sections will briefly introduce the three classes of small RNAs and what is known in terms of their biogenesis and function in mammals (see Fig. 6.2 for a summary).

1.2.1 miRNA and siRNA biogenesis and function

The miRNAs and endo-siRNAs can be distinguished from each other based on either their biogenesis or function; however, exceptions arise for both methods of definition. In general, miRNAs bind target mRNAs to inhibit translation and secondarily destabilize the transcript, whereas endo-siRNAs are thought to bind to target mRNAs and facilitate direct cleavage. This functional difference is due to the type of physical interaction between the small RNA and the target RNA: although complementary binding between the small RNA and mRNA target is involved in both cases, miRNAs

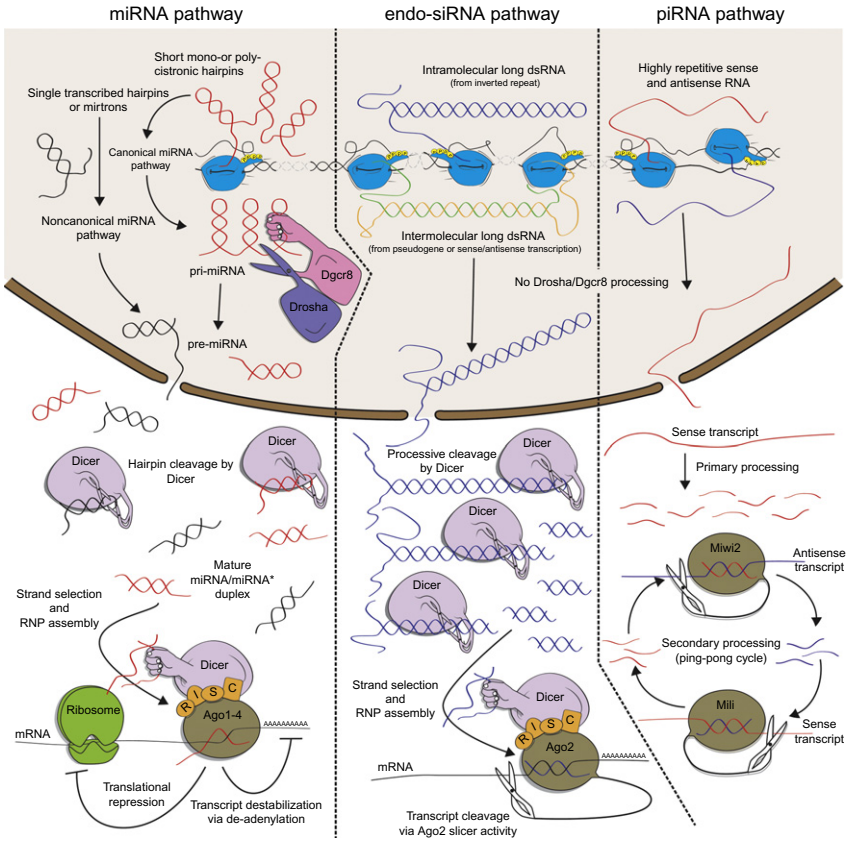

Figure 6.2 Schematic representation of small RNA biogenesis and function in mammals. (*miRNA pathway*) Canonical miRNAs are transcribed by RNA polymerase II as mono- or poly-cistronic hairpins known as primary miRNA (pri-miRNA) transcripts, which are subsequently cleaved by the microprocessor (Drosha/Dgcr8 complex) into pre-miRNAs. Non-canonical miRNAs are transcribed directly as single short hairpins, or they can be derived from intronic regions of a host mRNA, by-passing Microprocessor cleavage or other unknown endonuclease activity. After export to the cytoplasm, the RNase III-containing enzyme Dicer cleaves the hairpin, producing the mature miRNA/miRNA* duplex. The Dicer/miRNA complex then associates with Argonaute, where it guides one strand of the miRNA/miRNA* duplex into RISC with the complementary mRNA based on its seed sequence match. Target repression is generally achieved by inhibition of the translational machinery and/or destabilization of the transcript via deadenylation of the mRNA. (*endo-siRNA pathway*) Long double-stranded RNAs (dsRNAs) are the precursors to siRNAs. Whether long dsRNAs form from intramolecular or intermolecular complementary binding, they are exported from the nucleus without processing. In the cytoplasm, Dicer binds to and cleaves the dsRNA in a processive fashion, generating short dsRNA duplexes 21 nucleotides (21-nt) in length. The Dicer/siRNA complex guides one strand of the siRNA duplex into the Ago2-containing RISC based on perfect complementarity with the appropriate mRNA. Target repression is thought to be achieved by cleavage of the target transcript by Ago2, but little is known about endo-siRNA/target interactions in mammals. (*piRNA pathway*) Highly repetitive regions of the genome are transcribed to produce long sense and antisense RNAs. The sense transcript is exported, and a poorly understood primary processing event produces ~27-nt fragments that are then incorporated into a secondary processing event. Miwi2 forms a complex with the short sense RNAs bound to a long complementary antisense transcript, which is then cleaved into short RNAs that then

do not have a perfect match to their target transcript, whereas almost all siRNAs do. This results in a different mechanism by which target repression occurs: (1) an imperfect match (miRNA) results in a bulge in the duplex between the miRNA and its mRNA, leading to translational inhibition and protection from direct cleavage by endonucleases. Additionally, miRNA targeting promotes mRNA destabilization via decapping and shortening of the poly(A) tail (Fig. 6.2, left column); (2) a perfect match (siRNA) results in cleavage of the target transcript (Fig. 6.2, middle column; Doench, Petersen, & Sharp, 2003; Fabian, Sonenberg, & Filipowicz, 2010; Zeng, Yi, & Cullen, 2003). It should be noted that there are rare exceptions to this definition: miRNAs with a perfect match to their mRNA transcript are known to cleave targets in the same manner that siRNAs usually function (Yekta, Shih, & Bartel, 2004). It is also possible for siRNAs to bind imperfectly to their target transcripts, resulting in a bulge that prevents cleavage. For the purpose of this chapter, we will refer to miRNA function as an imperfect match with target transcripts resulting in translational inhibition and siRNA function as a perfect match with target transcripts and direct cleavage.

To better understand how miRNAs and siRNAs differ, it is important to distinguish their origins. There are two types of miRNAs, canonical and noncanonical. Both types are derived from either coding or noncoding RNA transcripts that contain hairpins (in coding RNAs, the miRNA component usually resides in the intron) (Cai, Hagedorn, & Cullen, 2004; Lee, Kim, et al., 2004). Canonical miRNAs derive from a primary miRNA (pri-miRNA), a long RNA that contains hairpins approximately 60–75 nt in length (Fig. 6.2, top left). The hairpins are recognized and cleaved at their base by a protein complex called the Microprocessor, which includes the RBP DGCR8 and the RNase enzyme DROSHA. The released hairpin is called the pre-miRNA, and it is exported to the cytoplasm by Exportin-5 for further processing (Bohnsack, Czaplinski, & Gorlich, 2004; Denli, Tops, Plasterk, Ketting, & Hannon, 2004; Gregory et al., 2004). Noncanonical miRNA production circumvents DROSHA-DGCR8 processing; instead, pre-miRNAs are produced from cleavage by other endonucleases or generated directly from the transcription of a single, short hairpin (Babiarz, Ruby, Wang, Bartel, & Blelloch, 2008; Okamura, Hagen, Duan, Tyler, & Lai, 2007; Ruby, Jan, & Bartel, 2007). Regardless of canonical or noncanonical origin, pre-miRNAs are exported to the

associate with Mili to cleave more sense transcripts, producing a positive feedback loop known as the ping-pong cycle. The ultimate functional mechanism of piRNAs is poorly understood in mammals, but it is possible that PIWI-related proteins associate with piRNAs and recruit chromatin-modifying machinery to specific regions of the genome to promote epigenetic changes that repress expression of mobile genetic elements.

cytoplasm where the RNase III enzyme, DICER, cleaves them into a single 18–25 nt dsRNA (Bernstein, Caudy, Hammond, & Hannon, 2001; Ketting et al., 2001). At this point, one strand of the duplex miRNA will associate with a host of proteins known as the RNA-induced silencing complex (RISC, described below) (Fig. 6.2, bottom left). Correct strand specificity can be influenced by DICER association with TRBP (Chendrimada et al., 2005; Gregory, Chendrimada, Cooch, & Shiekhattar, 2005). The extent to which TRBP is necessary for strand selection is unclear, however, as the knockout phenotype is mild (Zhong, Peters, Lee, & Braun, 1999) relative to the embryonic lethality of the *Dicer* knockout (Bernstein et al., 2003).

In contrast to miRNAs, endo-siRNAs arise from long dsRNAs (Fig. 6.2, top middle), either as long hairpins or long sense/antisense RNA pairs (duplexes). In metazoa, DICER binds at one end of the long dsRNAs and then cuts processively at approximately 21-nt intervals along the length of the dsRNA, producing multiple siRNAs (Chung, Okamura, Martin, & Lai, 2008; Czech et al., 2008; Ghildiyal et al., 2008; Kawamura et al., 2008; Okamura, Balla, Martin, Liu, & Lai, 2008). Therefore, DICER is necessary for the final cleavage event in the production of mature siRNAs, canonical miRNAs, and noncanonical miRNAs.

Similar to the case with miRNAs, one strand of the siRNA duplex associates with an Argonaute protein to form an active RISC. At this point, the RISC-loaded siRNA or miRNA binds to and regulates the translation and stability of its target mRNAs. For miRNAs, almost all target identification is facilitated by a 7-nt "seed" sequence found at positions 2 through 8 of the mature miRNA as well as by other less well-characterized features downstream of the seed (Grimson et al., 2007; Lewis, Shih, Jones-Rhoades, Bartel, & Burge, 2003). For almost all siRNAs, there is perfect complementation of the siRNA to the target transcript. Most miRNA target sites are found within the 3′UTR of mRNAs, and RBPs can bind to and alter secondary structure within the 3′UTR to enhance or inhibit the effects of small RNAs on target transcripts (Kedde et al., 2007, 2010); this often has profound biological significance. For example, in the case of zebrafish germ cells, the presence of the RBP Dazl is essential to ensure translation of germ cell-specific genes that are otherwise suppressed by miRNAs (Takeda, Mishima, Fujiwara, Sakamoto, & Inoue, 2009).

In mouse, there are four related Argonaute proteins (Ago1–4). Only AGO2 can lead to cleavage of a perfect target (Liu et al., 2004). Thus, siRNA target cleavage is dependent on AGO2. However, all four Argonaute proteins are highly redundant in terms of miRNA activity—loss of all four in ES cells is required to ablate miRNA activity, but reintroduction of any one of the four can

fully rescue miRNA activity (Su, Trombly, Chen, & Wang, 2009). This is in contrast to the situation in the fly where there is largely a division of labor among Ago proteins: Ago1 and Loquacious facilitate miRNA activity, while Ago2 and its partner R2D2 drive siRNA function (Ghildiyal & Zamore, 2009).

1.2.2 piRNA biogenesis and function

Three specific features distinguish piRNAs from miRNAs and endo-siRNAs: piRNAs are slightly longer (25–32 nt), do not require DICER for their processing (Fig. 6.2, right), and are expressed predominantly in the germline (Aravin et al., 2006; Houwing et al., 2007; Vagin et al., 2006; Watanabe et al., 2006). The complete mechanism by which piRNAs are produced and function remains unclear; however, most map to clusters in repeat sequences of the genome and are thought to defend against transposable elements (Klattenhoff & Theurkauf, 2008).

Much of what is known about piRNA biogenesis has been uncovered in the fly. The piRNA-interacting protein for which piRNAs were named, Piwi, was first identified in *Drosophila* as a mutation that caused defects in spermatogenesis (Lin & Spradling, 1997). PIWI stands for "P-element-induced wimpy testis." Since then, additional PIWI-like proteins have been discovered in *Drosophila*, including Aubergine (Aub) and Argonaute 3 (Ago3) (not to be confused with the mammalian Ago3, which functions in miRNA activity). All three of these proteins are highly expressed in the germline and associate with piRNAs (Carmell, Xuan, Zhang, & Hannon, 2002; Cox, Chao, & Lin, 2000; Harris & Macdonald, 2001; Parker & Barford, 2006). They are distantly related to the other Ago proteins and therefore form a subclade in the Ago family.

The biogenesis of piRNAs is poorly understood in mammals, although preliminary insights from the fly have been gained in recent years. Primary piRNA production in *Drosophila* involves the proteins Armitage, Zuchinni, and Yb (Saito et al., 2010; Siomi, Miyoshi, & Siomi, 2010). Long, single-stranded RNA transcripts are cleaved by these proteins and loaded onto Piwi/Aubergine to direct cleavage of transposon targets—these Aubergine-bound piRNAs represent class I piRNAs (Li et al., 2009; Malone et al., 2009; Saito et al., 2010). Piwi-mediated cleavage of the transposon target RNA generates class II piRNAs, which then associate with Ago3 and target more primary piRNA transcripts, thus creating a feedback loop known as the ping-pong model (Aravin, Sachidanandam, Girard, Fejes-Toth, & Hannon, 2007; Brennecke et al., 2007). These ping-pong-derived piRNAs are thought to be only one type of piRNA. More research is needed to understand how other piRNAs are generated from complex intergenic regions.

Similar to *Drosophila* Piwi, the mammalian orthologs *Mili*, *Miwi*, and *Miwi2* are required for spermatogenesis (Carmell et al., 2007; Deng & Lin, 2002; Kuramochi-Miyagawa et al., 2001). Mammalian piRNAs can be divided into the pachytene and prepachytene subtypes (Aravin et al., 2007). Prepachytene piRNAs (26–28 nt) are expressed in spermatogonia prior to meiosis and associate with the mouse PIWI-family proteins MILI or MIWI-2 before decreasing in levels at mid-pachytene. Pachytene piRNAs (29–31 nt) are expressed in the pachytene stage of meiosis and associate with MIWI until the cells reach the round spermatid stage (Aravin et al., 2007). It is thought that mouse pachytene piRNAs are generated by the primary processing pathway discovered in fly (Fig. 6.2, right), though their specific function remains unclear. Prepachytene piRNAs, in turn, are thought to defend against transposable elements because they are derived from repeat elements (Brennecke et al., 2007); their biogenesis and mechanism of action remain unknown.

An additional class of Piwi-associated small RNAs, known as the 21U-RNAs, has been discovered in *C. elegans* (Ruby et al., 2006). These RNAs also silence transposon activity and bind to piwi-like proteins but are synthesized via a different mechanism (Batista et al., 2008; Ruby et al., 2006; Wang & Reinke, 2008). Interestingly, 21U-RNA sequencing and transposon profiling data do not suggest direct silencing of transposons by 21U-RNAs. However, several recent reports have provided the first evidence that the 21U-RNAs direct the biogenesis of a second pool of small RNAs, termed 22G-RNAs. These are considered the "effector" components (which map to expressed transposons in *C. elegans*) and are thought to direct gene silencing, presumably through heterochromatin formation around the genomic loci that produce these transposons, sustaining a transgenerational silencing effect (Ashe et al., 2012; Bagijn et al., 2012; Lee et al., 2012; Shirayama et al., 2012).

2. SMALL RNAs IN GERM CELLS

Much of what has been learned about the functional roles of small regulatory RNAs in mammals has come from studies of loss-of-function mutants blocking the biogenesis or function of the different classes. In particular, mutants for *Drosha*, *Dgcr8*, *Dicer*, the Argonaute proteins, and the Piwi-related proteins have uncovered numerous roles for small RNAs during development. Table 6.1 provides a summary of knowledge gained from genetic models where individual small RNAs or the proteins important for their biogenesis and/or function have been depleted and analyzed in

Table 6.1 Summary of the genetic evidence for a role of small RNAs in germ cell development

Model organism	Mutation	small RNAs affected	Phenotype	Reference
Mus musculus (Mouse)	Dicer1 (TNAP-Cre)	miRNAs and siRNAs	Gonad colonization: slower proliferation during early differentiation	Hayashi et al. (2008) and Maatouk, Loveland, McManus, Moore, and Harfe (2008)
			Spermatogenesis: early proliferation and later sperm morphology and motility defects	Hayashi et al. (2008) and Maatouk et al. (2008)
	Dicer (Amh2-Cre)	miRNAs and siRNAs	Spermatogenesis: complete testicular degeneration, defects starting five days after birth	Papaioannou et al. (2009) and Kim et al. (2010)
	Dicer (Amhr2-Cre)	miRNAs and siRNAs	Oogenesis: larger pool of primordial follicles, premature folliculogenesis, oocyte migration defects from oviduct to uterus, shorter uterine horns and oviductal cysts, female infertility	Hong, Luense, McGinnis, Nothnick, and Christenson (2008), Nagaraja et al. (2008), Gonzalez and Behringer (2009) and Lei, Jin, Gonzalez, Behringer, and Woodruff (2010)
	Dicer1 (Zp3-Cre)	miRNAs and siRNAs	Oogenesis: arrest at meiosis I with a disorganized spindle and chromosome segregation defects	Murchison et al. (2007)
	Dgcr8 (Zp3-Cre)	miRNAs	No phenotype[a]	Suh et al. (2010)
	Drosha (Stra8-Cre)	miRNAs	Spermatogenesis: depletion of spermatocytes and spermatids	Wu et al. (2012)
	LIN-28	let-7 gain-of-function	PGC specification: fewer stella-positive PGCs are specified, rescued by Blimp-1 overexpression	West et al. (2009)

Continued

Table 6.1 Summary of the genetic evidence for a role of small RNAs in germ cell development—cont'd

Model organism	Mutation	small RNAs affected	Phenotype	Reference
	miR-290 cluster	miR-290–295	PGC migration: PGCs migrate off course	Medeiros et al. (2011)
			Oogenesis: the few PGCs that colonize the gonad die off, making females sterile	Medeiros et al. (2011)
	miR-17–92 cluster	miR-17–92	No phenotype[a]	Ventura et al. (2008)
	Ago2 (TNAP-Cre)	siRNAs	No phenotype[a]	Hayashi et al. (2008)
	Ago2 (Zp3-Cre)	siRNAs	Oogenesis: mature oocytes with disorganized spindles and chromosome segregation defects	Kaneda, Tang, O'Carroll, Lao, and Surani (2009)
	Miwi	piRNAs	Spermatogenesis: spermatogenic arrest at the beginning of the round spermatid stage (meiosis II) and sterile males; females are fertile	Deng & Lin (2002)
	Mili	piRNAs	Spermatogenesis: spermatogenic arrest at zygotene to early pacytene (meiosis I), sterile males; females are fertile	Kuramochi-Miyagawa et al. (2004)
	Miwi2	piRNAs	Spermatogenesis: spermatogenic arrest in early prohase (meiosis I), sterile males; females are fertile	Carmell et al. (2007)
Drosophila melanogaster (Fly)	Dicer1	miRNAs	PGC specification: Vasa-positive PGCs do not form in mutants	Megosh, Cox, Campbell, and Lin (2006)
	Loquacious	miRNAs	Oogenesis: necessary for GSC maintenance	Forstemann et al. (2005) and Park, Liu, Strauss, McKearin, and Liu (2007)
	Ago1	miRNAs	Oogenesis: necessary for GSC maintenance	Yang, Chen, et al. (2007), Yang, Duan, et al. (2007) and Neumuller et al. (2008)

	R2D2	miRNAs[b]	Oogenesis: necessary for stalk and follicle cell formation and, thus, normal oogenesis	Kalidas et al. (2008)
	Dicer2	siRNAs	No phenotype[a]	Hatfield et al. (2005) and Megosh et al. (2006)
	Ago2	siRNAs	No phenotype[a]	Okamura, Ishizuka, Siomi, and Siomi (2004)
	Piwi	piRNAs	PGC specification: Vasa-positive PGCs do not form in mutants	Megosh et al. (2006)
			Gametogenesis: necessary for GSCs of both sexes to be maintained, but not for further differentiation	Lin and Spradling (1997), Cox et al. (1998), and Szakmary, Cox, Wang, and Lin (2005)
	Aubergine	piRNAs	PGC specification: pole cells are lost in mutants	Schmidt et al. (1999), Harris and Macdonald (2001), and Thomson, Liu, Arkov, Lehmann, and Lasko (2008)
	Ago3	piRNAs	Oogenesis: females are sterile	Li et al. (2009)
			Spermatogenesis: subfertile due to misregulation of GSC mainteance	Li et al. (2009)
Danio rerio (Zebrafish)	Dicer1	miRNAs and siRNAs	No phenotype[a]	Giraldez et al. (2005)
	Ziwi	piRNAs	Early gametogenesis: loss of germ cells due to apoptosis	Houwing et al. (2007)
	Zili	piRNAs	Early gametogenesis: loss of germ cells due to apoptosis	Houwing, Berezikov, and Ketting (2008)

Continued

Table 6.1 Summary of the genetic evidence for a role of small RNAs in germ cell development—cont'd

Model organism	Mutation	small RNAs affected	Phenotype	Reference
Caenorhabditis elegans (Nematode)	Dicer1	miRNAs and siRNAs	Gametogenesis: abnormal oocyte morphology and migration through the spermatheca	Knight and Bass (2001)
	Ego-1	siRNAs	Gametogenesis: defects in mitotisis, premature, meiosis, errors in meiotic recombination	Smardon et al. (2000) and Knight and Bass (2001)
	Prg-1	21U-RNAs (piRNAs)	Spermatogenesis: mutant sperm exhibit extensive defects in activation and fertilization	Batista et al. (2008) and Wang and Reinke (2008)

[a]There is no germline phenotype as reported by the correlating reference; there may be a somatic phenotype.
[b]R2D2 normally associates with Ago2 in the siRNA pathway, but depletion causes a phenotype in the germline due to effects on the miRNA pathway.
All mutations are whole organism loss-of-function experiments, unless a conditional deletion was analyzed (indicated by the type of Cre recombinase used in parentheses).

the germline. Follow-up studies involving small RNA profiling and individual small RNA knockdown or introduction have further refined our knowledge of the role these small molecules play in critical germ cell fate decisions. These following sections will explore our current understanding of small RNAs at each stage of germ cell development: specification and migration, gonad colonization and differentiation, and gametogenesis.

2.1. Small RNAs in PGC specification and migration

In mouse, mutations deleting *Dicer1* are embryonic lethal around the time PGCs are specified, revealing a role for miRNAs and/or siRNAs in early development but leaving the question of PGC specification unresolved due to the timing of the defect (Bernstein et al., 2003). To better understand the role of small RNAs at the stage of PGC specification, various groups have utilized expression studies, directed miRNA or RBP knockouts, and generated *in vitro* models to uncover a role for miRNAs and the RBPs that regulate them.

One prominent miRNA, let-7, has been implicated in PGC specification (West et al., 2009). Mouse PGCs are induced by BMP4 from somatic cells to express *Prdm1* (*Blimp1*) and *Prdm14*, which suppress Hox genes and facilitate the reacquisition of pluripotency (Ohinata et al., 2005; Robertson et al., 2007; Vincent et al., 2005). *Prdm1* is suppressed in other tissues by the widely expressed miRNA let-7 via binding sites in its 3′UTR (Nie et al., 2008). Let-7 itself is antagonized by the activity of the RNA-binding protein LIN28, which is expressed in PGCs. LIN28 can bind to pre-let-7 and recruit TUT-4 to uridylate the 3′ end, blocking further processing by DICER (Heo et al., 2008; Newman, Thomson, & Hammond, 2008; Piskounova et al., 2008; Rybak et al., 2008; Viswanathan, Daley, & Gregory, 2008). The knockdown of LIN28 decreases the number of PGCs (West et al., 2009). Moreover, loss of LIN28 in mouse ES cells differentiated into PGC-like cells results in loss of *Prdm1* expression and a failure to maintain a germ cell-like state. Alternatively, overexpression of LIN28 results in more *in vitro* PGCs and more Stella-positive PGCs in chimeric embryos (West et al., 2009). Thus, LIN28 plays a critical role in PGC specification by preventing let-7 from suppressing *Prdm1* expression. However, it should be noted that LIN28 has additional targets (Jin et al., 2011; Peng et al., 2011), as does let-7 (Melton, Judson, & Blelloch, 2010), which are likely to play additional downstream roles in PGC specification.

LIN28 is not the only RBP with likely roles in regulating miRNA function in PGCs. The RBP *Dead end 1* (*Dnd1*) is essential in early PGC

development (Youngren et al., 2005). One known function of DND1 is to block miRNA function via binding mRNA transcripts and blocking miRNA access (Kedde et al., 2007). The exact targets of DND1 at the time of PGC specification are unknown. Loss of *Dnd1* does not specifically affect PGC specification or the path of migration but does affect the ability of PGCs to proliferate and survive during migration such that only a small number colonize the gonad (Cook, Coveney, Batchvarov, Nadeau, & Capel, 2009; Sakurai et al., 1995). These data suggest a critical role for DND1 in blocking the activity of specific miRNAs that target transcripts promoting growth and survival.

Profiling experiments have revealed high expression of certain miRNAs during PGC migration, including the miR-17–92 and miR-290–295 clusters, which promote cell cycle and maintain embryonic stem cell pluripotency (Hayashi et al., 2008; Wang et al., 2008). Deletion of the miR-17–92 cluster does not appear to affect germ cell development, although it was not carefully evaluated as mutants die soon after birth (Ventura et al., 2008). In contrast, deletion of the miR-290–295 cluster results in migration defects such that a significantly reduced number of PGCs colonize the gonads (Medeiros et al., 2011). Males remain fertile, suggesting no essential role for the miR-290–295 cluster during adult spermatogenesis. Females, however, fail to compensate for the loss of PGCs after specification and migration, and they are sterile secondary to premature ovarian failure (Medeiros et al., 2011).

Strikingly, in zebrafish, deletion of *dicer* does not result in any germ cell phenotype (Giraldez et al., 2005). However, miRNAs are still important in zebrafish for regulating maternally deposited mRNAs, especially those required for PGC specification (Giraldez et al., 2005, 2006). In particular, miR-430, one of the most highly expressed miRNAs, is necessary to prevent germ plasm RNAs from being expressed in somatic cells. Transcripts that specify the PGC fate in zebrafish are not limited to the germ plasm but are also expressed in somatic cells. Many of these transcripts (*nanos*, *tdrd7*, *dead end*) are miR-430 targets and are thus destabilized and translationally repressed in the somatic compartment (Blaser et al., 2005; Giraldez et al., 2006; Koprunner et al., 2001; Mishima et al., 2006). These transcripts are ectopically expressed in somatic cells where *dicer* is mutated and miR-430 levels are diminished (Mishima et al., 2006). Surprisingly, miR-430 is also expressed in germ cells. How is miR-430 kept from inhibiting expression of these critical germ plasm determinants in the cells that become PGCs? The answer involves RBPs. The zebrafish *Dnd1* ortholog binds to the 3′UTR of

germ plasm transcripts and blocks miR-430 activity (Kedde et al., 2007; Mickoleit, Banisch, & Raz, 2011; Slanchev et al., 2009), similar to what is suspected to occur in the mouse. In the absence of *dnd* in zebrafish, PGCs never become motile: they do not polarize or form pseudopodia, and they begin to undergo apoptosis (Weidinger et al., 2003). Zebrafish Dazl also plays a role in preventing the action of miR-430 in PGCs but through a different mechanism than Dnd. Dazl counteracts miR-430 activity by promoting translation of target transcripts via poly(A) tail elongation (Takeda et al., 2009).

Together, these findings highlight the role RBPs play in mitigating the function of miRNAs and emphasize the need to understand the cellular context when determining whether small RNAs will significantly affect target expression. In fact, a recent report attempting to define the minimal criteria for normal PGC morphology and motility during PGC migration in zebrafish found that a delicate balance between miR-430 and the RBP Dnd is necessary to generate a threshold of protein expression of target transcripts that encode factors regulating myosin contractility, cell adhesion, and cell cortex properties (Goudarzi et al., 2012).

C. elegans mutants lacking EGO-1 and Dicer1 (proteins necessary for endo-siRNA synthesis) only showed defects after germline specification (Knight & Bass, 2001; Smardon et al., 2000). Similarly, in fly, Dicer2 and Ago2 (both required for siRNA activity) are dispensable for germline specification and maintenance, indicating that endo-siRNA activity is not necessary (Hatfield et al., 2005; Lee, Nakahara, et al., 2004; Megosh et al., 2006; Okamura et al., 2004). However, loss of Piwi, dFmrp (a component of the RISC complex), and Dicer1 (required for miRNA activity) leads to pole plasm defects and reduced PGC numbers (Megosh et al., 2006). Interestingly, overexpression of Piwi increases Oskar and Vasa expression leading to a corresponding increase in PGC number, suggesting a central role for piRNAs (Megosh et al., 2006). Deletion of Aubergine, another PIWI homolog associated with polar granules, is also necessary for the normal translation of Oskar and ultimately the formation of PGCs (Harris & Macdonald, 2001; Thomson et al., 2008). Together, these findings indicate roles for piRNAs and miRNAs in PGC specification.

How piRNAs function in the early germline is unclear, though there are some reports that piRNAs may target transcripts rather than transposons. Normal *nanos* deadenylation in the anterior end of the embryo is facilitated by piRNA activity in the *nanos* 3′-UTR (Rouget et al., 2010). When piRNA-induced regulation is impaired by Aubergine depletion, *nanos*

deadenylation decreases, and anterior defects are observed (Rouget et al., 2010). In addition to *nanos*, piRNAs might also target *vasa* transcripts, which encode another RBP essential for early germ cell development (Nishida et al., 2007). The only evidence supporting a role for piRNAs in PGC specification and migration in vertebrates comes from a recent report characterizing a deletion of the medaka (Japanese killifish) homolog of Piwi, *Opiwi*. Loss of *Opiwi* results in fewer PGCs being specified and major migration defects (Li, Hong, Gui, & Hong, 2012).

2.2. Small RNAs in PGC gonad colonization and early differentiation

Across metazoa, PGCs migrate to the gonad and then begin to associate with somatic cells as they commit to a sex-specific fate. Conditional deletion of critical components of small RNA biogenesis and function has been used to dissect the role of these pathways at this stage of germ cell development. *TNAP-Cre* deletion of *Dicer1* in mice at a time when PGCs are just arriving in the gonad results in reduced proliferation and differentiation defects in male, but not female, mice (Hayashi et al., 2008). These defects are likely a result of diminished miRNA activity, as the *TNAP-Cre Ago2* conditional deletion shows no phenotype (Hayashi et al., 2008). That is, deletion of *Ago2* is expected to ablate all siRNA function, while the three other Ago proteins, if expressed, can maintain miRNA function.

Sex differentiation of gametes occurs upon their arrival at the gonad. miRNA expression profiling of PGCs colonizing the gonad reveals differential expression patterns of miRNAs between the sexes: let-7, miR-125a, and miR-9 are upregulated in males (Hayashi et al., 2008), possibly contributing to their sex-specific differentiation and exit from the cell cycle; female germ cells express miR-29b (Takada, Berezikov, Choi, Yamashita, & Mano, 2009), which may be important for meiotic entry. It is believed that miR-29b targets *Dnmt3a* and *Dnmt3b* to facilitate changes in the methylation status of the female germline (Takada et al., 2009). The miR-290–295 cluster, which is important in PGC migration, is dispensable for male germ cell commitment and progression, but females lacking this cluster suffer germ cell loss and premature ovarian failure (Medeiros et al., 2011). It remains unclear whether female sterility is secondary to the premature ovarian failure or whether the miR-290–295 cluster plays a direct role in female germ cell development.

RBPs that regulate miRNA function in early development also have a role in PGC colonization of the gonad. In mouse, male germ cells enter

mitotic arrest and downregulate markers of pluripotency as they upregulate cell cycle inhibitors of the G1/S transition such as *p21* (*Cdkn1a*) and *p27* (*Cdkn1b*) (Western et al., 2008). The RBP DND1 binds the 3′UTR of these transcripts and protects them from miRNA-mediated inhibition by miR-221/222 such that mitotic arrest can occur—mutants of *Dnd1* lose expression of *p21* and *p27*, maintain markers of cell cycle and pluripotency, and develop teratomas on certain genetic backgrounds (Cook, Munger, Nadeau, & Capel, 2011; Kedde et al., 2007; Youngren et al., 2005). Alternatively, the RBP PUM1, expressed in many tissues including germ cells, can alter the secondary structure of target transcripts to enhance the binding of miR-221/222 and the subsequent inhibition of translation (Kedde et al., 2010), though it is unclear whether this particular role is essential for germ cell development. These examples reveal that the coordinate expression (and timing) of multiple RBPs is essential to promote or antagonize miRNA activity of target transcripts that define a complex cell fate/identity.

In the fly, PGCs, together with somatic cells, form a GSC niche where the GSCs divide asymmetrically to produce a daughter stem cell and a progenitor cell that proceeds through gametogenesis. The loss of Dicer-1 results in a delay of the G1/S checkpoint in GSCs, in part, due to an increase in the cell cycle inhibitor Dacapo (a p21/p27 homolog) (Hatfield et al., 2005). Further analysis of mutants revealed that insulin signaling drives expression of miR-7, miR-278 m and miR-309, which target *dacapo* and are essential for normal GSC division (Yu et al., 2009). In *dicer-1* mutants, GSCs eventually differentiate and are lost (Jin & Xie, 2007). Similar GSC phenotypes were observed in other miRNA pathway mutants including *loquacious*, *ago1*, and *dfmrp* (Forstemann et al., 2005; Neumuller et al., 2008; Park et al., 2007; Pek, Lim, & Kai, 2009; Yang, Chen, et al., 2007; Yang, Duan, Chen, Wang, & Jin, 2007). For example, dFMRP is an RBP that binds to the miRNA *bantam*, and this interaction is essential to maintain GSC self-renewal (Yang, Duan, et al., 2007; Yang et al., 2009). These studies support the idea that miRNAs play a crucial role in maintaining the GSC niche of the fly, regulating cell cycle and differentiation genes that are critical for early gametogenesis.

Another example of small RNAs regulating early differentiation of the GSC niche in the fruit fly is miR-184 in the female germline. miRNA-184 is expressed in female GSCs and regulates differentiation by controlling the level of expression of the Saxophone receptor (Iovino, Pane, & Gaul, 2009). Dpp (Decapentaplegic) from the niche signals through the Saxophone receptor to suppress Bam, a protein essential for initiating

gametogenesis (Xie & Spradling, 1998); thus, miR-184 suppresses Dpp signaling, resulting in more Bam and promoting differentiation (Iovino et al., 2009). Consequently, mutant miR-184 GSCs fail to differentiate because they never activate Bam signaling. Thus, the exquisite timing of Bam-mediated differentiation in the *Drosophila* germarium is dependent on the expression of miR-184 to prevent the niche from generating only GSCs.

Some miRNAs inhibit, rather than promote, normal GSC niche maintenance. In fly, the Piwi-associated nuage component, Maelstrom, drives GSC maintenance and self-renewal by blocking miRNA activity (Findley, Tamanaha, Clegg, & Ruohola-Baker, 2003; Soper et al., 2008). Maelstrom represses the transcription of miR-7, which targets *Bam*—loss of Maelstrom results in miR-7 repression of *Bam* and a failure to enter meiosis (Pek et al., 2009). Some proteins involved in GSC development prevent miRNA activity globally. For example, the RBP Vasa, which associates with the nuage, acts as an inhibitor of miRNA activity, albeit indirectly: Vasa binds to and promotes the expression of the *mei-P26* transcript, which in turn binds to and inhibits Ago1, resulting in downregulation of many miRNAs (Liu, Han, & Lasko, 2009; Neumuller et al., 2008). Importantly, upregulation of Mei-P26 occurs in 16 cell cysts and not GSCs themselves (Neumuller et al., 2008), thus theoretically preserving miRNA activity in GSCs. Therefore, whether through direct interaction with individual miRNAs or indirect action on the proteins regulating miRNA biogenesis and/or function, RBPs and other proteins cooperate with miRNAs in normal GSC maintenance and differentiation in the fly.

The requirement for miRNAs and siRNAs in early germline development differs across systems. In zebrafish, for example, loss of Dicer does not affect the ability of animals to produce mature sperm and eggs (Giraldez et al., 2005). However, *C. elegans* mutants lacking *dicer-1* show severe defects in spermatogenesis (Knight & Bass, 2001). Consistent with a role for siRNAs in spermatogenesis, nematodes lacking the RNA-dependent RNA Polymerase (RdRP) homolog EGO-1 (required for siRNA processing and RNAi) display severe defects in both oogenesis and spermatogenesis that result in infertility due to defects in mitosis, premature meiosis, and errors in meiotic recombination (Smardon et al., 2000). As an RdRP family member, EGO-1 is believed to be important for the secondary step of siRNA synthesis in *C. elegans* (Gent et al., 2009).

Drosophila is one of the few model organisms for which a role of piRNAs has been determined in early germline development. In the fly, piRNAs have been reported to have a role in PGC colonization of the gonad and

early differentiation. Somatic and germline cells of both sexes express Piwi proteins, which are necessary for piRNA activity. GSCs are lost in *piwi* mutant flies of both sexes, and Piwi overexpression in female somatic cells results in an increased rate of GSC division and number (Cox et al., 1998; Szakmary et al., 2005). Aubergine and Ago3 are also required for fertility in both sexes (Li et al., 2009; Lin & Spradling, 1997; Schmidt et al., 1999). Piwi proteins and piRNAs also play a critical role in mouse germ cells, but at a later stage of differentiation, as described below.

2.3. Small RNAs in spermatogenesis

In mouse, the miRNA and piRNA pathways are essential for spermatogenesis (Carmell et al., 2007; Hayashi et al., 2008; Maatouk et al., 2008; Unhavaithaya et al., 2009). The proteins DICER, AGO1, and DROSHA, as well as individual miRNAs, localize to the chromatoid body, a center for RNA processing that is specific to male germ cells during spermatogenesis (Gonzalez-Gonzalez, Lopez-Casas, & del Mazo, 2008; Kotaja et al., 2006; Kotaja & Sassone-Corsi, 2007).

Loss of *Dicer* in mouse PGCs results in early male germ cell proliferation defects, impaired spermatogenesis, and defective sperm morphology and motility (Hayashi et al., 2008; Maatouk et al., 2008). These defects are due to problems in miRNA activity because *Ago2* deletion (which ablates siRNA function while leaving miRNA activity intact) is indistinguishable from wild type with regard to germ cell proliferation, differentiation, and meiotic progression (Hayashi et al., 2008). Further, deletion of Drosha (required for canonical miRNA, but not endo-siRNA, biogenesis) in spermatocytes using a *Stra8-Cre* results in loss of spermatocytes and spermatids and eventual male sterility (Wu et al., 2012).

Roles for some individual miRNAs in spermatogenesis have been characterized. The miR-17–92 cluster, which targets *E2F1*, protects meiotic cells from apoptosis (Novotny et al., 2007). miR-122a represses *Tnp2* in postmeiotic germ cells (Yu, Raabe, & Hecht, 2005), while TRANSLIN, another RBP, binds to miR-122a and promotes its stability in late-stage spermatids (Yu & Hecht, 2008). Thus, miR-122a is critical as part of a coordinated effort to repress *Tnp2* in differentiating spermatocytes. miRNA-34c is highly expressed in male germ cells during late spermatogenesis, and its overexpression in an *in vitro* model results in upregulation of germ cell-specific genes (Bouhallier et al., 2010). Alternatively, miR-18 of the miR-17–92 cluster targets *Hsf2*, a germ cell transcription factor that is essential for normal regulation of spermatogenesis (Bjork, Sandqvist, Elsing,

Kotaja, & Sistonen, 2010). Another clue that miRNAs are important in spermatogenesis comes from miRNAs encoded by the X-chromosome. Although all X-linked mRNA genes studied to date have been shown to be transcriptionally silent during spermatogenesis, many X-linked miRNAs circumvent this transcriptional quiescence (Song et al., 2009). It remains unclear what specific function these miRNAs perform and whether they are essential for normal spermatogenesis.

Some RBPs promote the action of miRNAs by binding target transcripts and altering secondary structure to permit the binding of complementary miRNAs. For example, the RBP Mouse Vasa Homolog interacts with Dicer and is thought to be the germ cell-specific helicase for some miRNA pathways during spermatogenesis (Kotaja et al., 2006). While promoting miRNA activity is essential at some stages of spermatogenesis, suppression of miRNA activity at other stages is also critical. Of particular interest is the RBP LIN28, known for its inhibition of the let-7 family of miRNAs; LIN28 is expressed in human and nonhuman primate SSC populations (Aeckerle et al., 2012), suggesting that blocking let-7 may be essential to maintain an adult population of SSCs.

Expression of miRNAs in somatic cells is also important for normal regulation of spermatogenesis. In mouse, deletion of *Dicer1* in Sertoli cells (one of the main supporting cell lineages of the testis) results in major gene expression changes within the testis (Papaioannou et al., 2011) and eventually complete testicular degeneration, with defects manifesting about 5 days after birth (Kim et al., 2010; Papaioannou et al., 2009). This suggests a critical role for miRNAs in regulating Sertoli cell influence on germ cells, most likely through effects on paracrine signaling. It is possible that miRNAs regulate hormonal signaling, which is also important for spermatogenesis. Hormone suppression during spermiation results in an increase in over 20 miRNAs in Sertoli cells (Nicholls et al., 2011). This suggests that long-range hormonal control of male germ cell differentiation might be facilitated in part by miRNA activity.

Another great example of miRNA function in somatic cells regulating spermatogenesis comes from a recent report in the fly (Toledano, D'Alterio, Czech, Levine, & Jones, 2012). In *Drosophila*, male GSCs exhaust over time due to loss of signaling from the somatic hub cells. Specifically, loss of the self-renewing factor Unpaired in hub cells results in decreased JAK-STAT signaling and eventual loss of GSCs (Boyle, Wong, Rocha, & Jones, 2007). Normally, the RBP Imp binds to and promotes translation of Unpaired, sustaining normal JAK-STAT signaling for GSC maintenance.

However, the *Imp* transcript has binding sites for the miRNA let-7 in its 3′UTR. Over time, expression of let-7 increases in hub cells and represses expression of *Imp*, causing a subsequent decrease in Unpaired protein and GSC exhaustion (Toledano et al., 2012). The aging effect can be rescued by maintaining expression of Imp in hub cells over time, demonstrating the critical balance between miRNAs and RBPs that regulate GSC development in the aging *Drosophila* niche.

While many studies have shown roles for miRNAs in mouse spermatogenesis, endo-siRNAs appear to be dispensable (Hayashi et al., 2008). However, in nematodes, several mutations in components of the RNAi pathway have implicated roles for siRNAs in spermatogenesis (Gent et al., 2009; Han et al., 2009; Ketting, Haverkamp, van Luenen, & Plasterk, 1999; Pavelec, Lachowiec, Duchaine, Smith, & Kennedy, 2009; Rocheleau et al., 2008; She, Xu, Fedotov, Kelly, & Maine, 2009; Smardon et al., 2000; Tabara et al., 1999). In particular, the 26G and 22G RNAs (primary and secondary endo-siRNAs produced specifically in *C. elegans*) are necessary to prevent defects in chromosome segregation during both mitosis and meiosis (Han et al., 2009). The 22G RNAs recruit chromatin-modifying factors that promote local histone modifications that influence the binding and organization of Condensins and Cohesins necessary for normal chromosome segregation (a distinct role for this special class of dsRNA), and 26G RNAs regulate gene expression via association with the AGO proteins Alg3 and Alg4 (Claycomb et al., 2009; Conine et al., 2010; Gu et al., 2009; Han et al., 2009; van Wolfswinkel et al., 2009). Why siRNAs appear to play a critical role in *C. elegans* spermatogenesis and not in mouse remains to be determined.

In contrast to endo-siRNAs, piRNAs play an essential role in mouse spermatogenesis. Deletion of *Mili*, *Miwi*, and *Miwi-2* (mammalian Piwi homologs) results in activation of retrotransposons in male germ cells, meiotic arrest, and eventual male sterility (Aravin et al., 2007; Carmell et al., 2007; Deng & Lin, 2002; Kuramochi-Miyagawa et al., 2004, 2008). These same reports suggest that piRISC (piRNA-RNA-induced silencing complex) may also influence the *de novo* methylation machinery that silences transposons, though this interaction may be independent of slicer activity because a *piwi* mutant lacking slicer activity in the fly can also direct methylation of transposons (Saito et al., 2010). Supporting the idea that Piwi proteins could be driving epigenetic changes in mice, loss of *Mili* or *Miwi2* results in defective DNA methylation of regulatory regions of retrotransposons, suggesting a specific role in *de novo* DNA methylation of transposable elements in male germ cells (Aravin et al., 2007, 2008; Carmell et al., 2007;

Kuramochi-Miyagawa et al., 2008). Further, Piwi proteins can recruit heterochromatin protein 1A to genomic loci (Brower-Toland et al., 2007; Pal-Bhadra et al., 2004). More research will be necessary to determine the full extent to which small RNAs are directing epigenetic changes in mouse spermatogenesis.

In mice, the activation of mobile genetic elements coinciding with the loss of components of the piRNA machinery results in genomic instability. For example, in mouse *Miwi-2* mutants, most cells arrest at the leptotene stage of meiosis, which correlates with defects in dsDNA break repair, consistent with a role for MIWI2 in normal meiotic recombination (Carmell et al., 2007; Kuramochi-Miyagawa et al., 2004). *Miwi* mutants suffer spermatogenic arrest at the beginning of the round spermatid stage (Deng & Lin, 2002), and loss of *Mili* leads to a block at the zygotene or early pachytene stages (Kuramochi-Miyagawa et al., 2004). All of these defects are consistent with the timing of expression for each of these proteins: *Mili* and *Miwi-2* mutants showing arrest in meiosis I (primary spermatocyte stage) and deletion of *Miwi* results in arrest following meiosis II (round spermatid stage) (Deng & Lin, 2002).

The piRNAs also play a role in spermatogenesis in other vertebrate and invertebrate model organisms. In zebrafish, loss of piwi homologs, Ziwi and Zili, results in activation of transposable elements and male sterility (Houwing et al., 2007). Ziwi mutant germ cells undergo apoptosis, leading to male infertility, and loss of Zili results in a failure of germ cells to differentiate to mature oocytes or sperm. In the fly, piRNAs target repetitive sequences of the protein-coding gene *stellate* to maintain male fertility (Aravin et al., 2001). In nematodes, loss of prg-1, a piwi-like protein that is localized to P-granules, results in a reduction in the expression of 21U-RNAs (a *C. elegans*-specific class of piwi-interacting small RNAs that are shorter than other piRNAs) during spermatogenesis, which correlates with multiple defects in sperm activation and fertilization (Batista et al., 2008; Wang & Reinke, 2008). These results clearly demonstrate a critical role for piRNAs and Piwi proteins in spermatogenesis. However, the full mechanism of action for the piRNA pathway in each context has yet to be fully elucidated.

2.4. Small RNAs in oogenesis

Gene regulation in maturing oocytes is critical to ensure reprogramming to a totipotent zygote. In the female mouse germline, all three types of small RNAs have been shown to be present by deep sequencing analysis (Tam et al., 2008; Watanabe et al., 2006, 2008). A role for small RNAs in oogenesis was demonstrated based on knockout studies of *Dicer1* and *Ago2*, where

loss of these proteins resulted in meiotic and spindle organization defects (Kaneda et al., 2009; Murchison et al., 2007; Tang et al., 2007; Watanabe et al., 2008). It was believed that both endo-siRNAs and miRNAs were playing a role in the *Dicer* knockout; so, it was a surprise to learn that loss of *Dgcr8* (which is only necessary for miRNA, not siRNA, biogenesis; Fig. 6.2, top) presented no defects in oocyte maturation, fertilization, or early development (Ma et al., 2010; Suh et al., 2010; Wang, Medvid, Melton, Jaenisch, & Blelloch, 2007). Together with reporter studies showing siRNA (but not miRNA) activity in oocytes (Ma et al., 2010), these findings suggest that endo-siRNAs are largely responsible for the *Dicer1* phenotype, and that although miRNAs are expressed, their activity is suppressed in maturing oocytes. Additionally, analyses of the small RNAs expressed in oocytes and the transcripts upregulated in *Dicer1* mutants have revealed expressed pseudogenes as a possible source of dsRNA for production of the endo-siRNAs (Tam et al., 2008; Watanabe et al., 2008).

What molecular events occur in the oocyte to block miRNA activity while leaving endo-siRNA activity intact? It is possible that the miRNA loss of function in oocytes and early embryos is secondary to the loss of processing bodies (P-bodies) that occurs at the GV stage and does not recover until the blastocyst stage (Flemr, Ma, Schultz, & Svoboda, 2010; Swetloff et al., 2009). However, it is equally likely that the loss of P-bodies, known sites of miRNA activity, is secondary to a primary block in miRNA function (Parker & Sheth, 2007). After fertilization, it is believed that miRNAs and siRNAs do not play a role in preimplantation development because zygotic deletions of *Dgcr8*, *Dicer*, and *Ago2* do not have a phenotype until after embryo implantation (Bernstein et al., 2003; Morita et al., 2007; Suh & Blelloch, 2011). Moreover, maternal deletion of *Dgcr8* has no phenotype until postimplantation, suggesting no essential role for maternal miRNAs throughout oocyte maturation and all of preimplantation development (Suh et al., 2010).

Similar to miRNAs, piRNAs are expressed in mouse oocytes but do not appear to be essential as deletion of the Piwi proteins does not result in an oocyte phenotype (Carmell et al., 2007; Deng & Lin, 2002; Kuramochi-Miyagawa et al., 2004). In flies, a lack of maternally deposited piRNAs that target transposons carried by the paternal genome can result in "hybrid dysgenesis," where sterile progeny result from DNA damage caused by transposon mobilization (Bourc'his & Voinnet, 2010; Brennecke et al., 2008). Fly piRNAs also control Fasciclin 3 (Fas3) levels in follicle cells of the ovaries to regulate oogenesis, and loss of Piwi results in a failure of the somatic cells

to intermingle with germ cells (Saito et al., 2009). In zebrafish, loss of Zili results in a failure of germ cells to differentiate to mature oocytes due to defects in chromosome segregation and early meiotic processes (Houwing et al., 2008; Houwing et al., 2007). These studies also revealed a crucial function for Zili independent of its function in transposon defense: in the presence of hypomorphic alleles of Zili where transposon transcripts were not ectopically upregulated, females remained sterile due to arrest in meiosis I (Houwing et al., 2008). More research will be needed to elucidate the mechanism by which the piRNA pathway affects female germline development and why these components are not essential for mouse oogenesis.

Oocyte development and maturation in mouse require intimate cellular interactions between the oocyte and soma. In mice, somatic cell loss of small RNAs leads to defects in oogenesis. Specifically, depletion of *Dicer1* in ovarian granulosa cells as well as the mesenchyme-derived cells of the oviduct and uterus causes female infertility (Gonzalez & Behringer, 2009; Hong et al., 2008; Lei et al., 2010; Nagaraja et al., 2008). Loss of *Dicer1* in granulosa cells, the cells surrounding the oocyte during development (utilizing the *Amhr2-Cre*), results in the establishment of a larger pool of primordial follicles in the neonatal ovary (Lei et al., 2010). Despite this larger pool of primordial follicles, these *Dicer1* mutants are infertile due to premature follicle maturation and loss of oocytes resulting from defects in granulosa cells. It is believed that the defects are due in part to a loss of miR-503, which normally targets Cyclin D2 (an important regulator of the cell cycle) as well as other genes important for granulosa cell proliferation and luteinization (Lei et al., 2010). The *Amhr2-Cre* also removes *Dicer1* from all Mullerian duct mesenchyme-derived tissues. Loss of *Dicer1* in these tissues leads to defects in ovulation due to a failure of the oocytes to migrate from the oviduct to the uterus (Hong et al., 2008). This defect may be secondary to structural defects, as the uterine horns are shorter and oviductal cysts develop (Gonzalez & Behringer, 2009; Nagaraja et al., 2008), presumably due to derepression of targets detrimental to normal reproductive tract development.

Like Sertoli cells of the testis, miRNAs present in mural granulosa cells also regulate the hormonal signals important for oogenesis. miR-132 and miR-212 are stimulated by human chorionic gonadotropin (which mimics endogenous LH activity) and are necessary for the downstream cAMP-dependent signaling events that result in normal meiotic progression (Fiedler, Carletti, Hong, & Christenson, 2008). Indeed, the link between miRNAs and hormone signaling has also been validated in human primary granulosa cell cultures, where a screen for miRNAs that affect expression

and release of testosterone and estradiol found that overexpression of more than 50 different miRNAs had a significant effect (Sirotkin, Ovcharenko, Grossmann, Laukova, & Mlyncek, 2009). Follow-up studies found that some miRNAs directly affect proliferation and apoptosis (Sirotkin, Laukova, Ovcharenko, Brenaut, & Mlyncek, 2010), though the direct targets through which these miRNAs act remain to be determined. Discovering the mechanism by which miRNAs mediate hormone signals *in vivo* will require more research.

Small RNA regulation of the soma is also crucial in fly oogenesis. *Drosophila* somatic stem cells (SomaSCs) in the ovary lacking Dicer1 failed to self-renew, which resulted in defective follicle cell growth and proliferation (Jin & Xie, 2007). Moreover, SomaSCs lacking Loquacious and Ago1 (affecting the miRNA pathway) resulted in an abnormally developed germarium (Forstemann et al., 2005; Park et al., 2007; Yang, Chen, et al., 2007). Further supporting a role for miRNAs in SomaSCs, SomaSC deletion of *belle*, another important component for RNAi in Drosophila S2 cells, resulted in GSC cell cycle and maturation defects due to misregulated timing of Notch activity in follicle cells (Deng, Althauser, & Ruohola-Baker, 2001; Lopez-Schier & St Johnston, 2001; Poulton et al., 2011). Specifically, miRNA targeting of the Notch ligand Delta regulates the timing of Notch expression in follicle cells, which then promotes normal GSC development (Poulton et al., 2011). Another role for miRNAs in the follicle cells of the fly ovary has also been uncovered in R2D2 mutants. R2D2, which is normally involved in siRNA production through an interaction with Dicer2, has also been found to bind Dicer1, indicating a role for this protein in the miRNA pathway. A loss of R2D2 results in follicle and stalk cell formation defects, leading to abnormal oogenesis and lower fertility (Kalidas et al., 2008). Together, these studies have revealed a profound impact of miRNAs in oogenesis and reproductive function through small RNA expression in somatic tissues and subsequent regulation of ovulation and early embryo development.

3. SMALL RNAS IN GERM CELL TUMOR FORMATION

Misregulation of germ cell development at any stage (specification, migration, gonad colonization, differentiation) can have disastrous outcomes, including abnormal differentiation and proliferation resulting in tumor formation. The latent potential of germ cells to produce tumors is evident by their ability to form embryonic germ cells (EGCs) when migratory PGCs are explanted to culture and placed under the influence of a

specific cocktail of signals (Fig. 6.1B; Cheng et al., 1994; Donovan & de Miguel, 2003; Labosky, Barlow, & Hogan, 1994). EGCs are very similar to embryonic stem cells (ESCs) in their developmental potential, and the PGCs from which EGCs are derived demonstrate this potential when they develop into various types of germ cell tumors (GCTs). Small RNAs and the RBPs that regulate them in normal germline development also have critical roles in the formation of GCTs. The following section will briefly highlight known roles of small RNAs in both controlling and promoting the development of GCTs.

3.1. miRNAs, siRNAs, and piRNAs in GCTs

Many of the processes that small RNAs regulate during the germ cell life cycle are linked to tumorigenesis. In the fly, Mei-P26 binds Ago1 and reduces miRNA levels in ovarian GSCs, resulting in restricted growth and proliferation (Neumuller et al., 2008). Loss of *mei-P26* results in expansion of the transit amplifying cell pool, unrestricted proliferation, and eventually ovarian tumor formation. The RBP Vasa normally promotes translation of *mei-P26* in the germline (Liu et al., 2009), suggesting that aberrant function of Vasa may also cause tumor formation. Thus, balancing levels of miRNA activity in the *Drosophila* female germline is essential to prevent the unchecked growth and proliferation of a tumor.

Similarly, in mouse, miRNA activity can promote tumor development. The RBP DND1 mitigates miRNA activity and regulates germ cell development at multiple stages (Cook et al., 2009, 2011; Kedde et al., 2007; Youngren et al., 2005). Loss of *Dnd1* in mouse results in miRNA suppression of cell cycle inhibitors that normally facilitate differentiation of male PGCs such that uncontrolled growth and proliferation gives rise to full blown teratomas in a substrain of mice (Cook et al., 2011; Stevens, 1973; Youngren et al., 2005). A screen for mutations in *Dnd1* in human testicular germ cell tumors (TGCTs), however, revealed only one patient with a detrimental mutation out of more than 260 TGCT samples, indicating alternative pathways of cell cycle derepression that potentially act through miRNA misregulation (Linger et al., 2008).

Other studies have linked small RNAs and RBPs regulating small RNAs with human GCT models. High levels of miR-372/373 are associated with human teratocarcinomas (Voorhoeve et al., 2007). The human miR-371–373 cluster is homologous to the mouse miR-290–295 (Houbaviy, Murray, & Sharp, 2003) and miR-17–92 clusters, which are highly upregulated in migrating PGCs and ESCs (Hayashi et al., 2008; Wang

et al., 2008). These human miRNAs are known to target *Lats2*, which is expressed in germ cells and is important for maintaining genomic stability; intriguingly, miRNA suppression of *Lats2* in humans can be relieved by the RBP DND1 (Kedde et al., 2007). This suggests that miRNAs and the RBPs that regulate them control sets of transcripts that work within common molecular pathways affecting broad cellular processes (Keene, 2007). To determine what other miRNAs and pathways might be involved in GCT development, a high-throughput miRNA qPCR screen in human samples confirmed elevated levels of the miR-371 cluster in nonseminomatous GCTs and revealed a differential expression pattern for 156 other miRNAs in various testicular tumors (Gillis et al., 2007).

Some RBPs that directly suppress miRNA activity are associated with tumor progression. For example, LIN28, which directly inhibits the activity of the let-7 family of miRNAs and thereby promotes pluripotency and self-renewal in ESCs, is overexpressed in malignant human GCTs, but not benign tumors or normal testicular tissue (West et al., 2009). Additionally, LIN28 is a strong marker for ovarian primitive GCTs (Xue, Peng, Wang, Allan, & Cao, 2011). Inhibition of LIN28 activity in human epithelial ovarian cancer cells resulted in significant reductions in cell growth and survival, providing corroborative evidence that LIN28 acts directly as an oncogene (Peng, Maihle, & Huang, 2010).

The piRNA pathway may also be involved in testicular tumors. Another TGCT sample study in human tissues revealed overexpression of the piRNA-binding protein HIWI (homolog of Piwi) in a majority of seminomas, but not nonseminomas or spermatocytic seminomas (Qiao, Zeeman, Deng, Looijenga, & Lin, 2002). The extent to which piRNA misregulation plays a role in GCT development remains unclear.

4. CONCLUSION

Small RNAs have emerged as critical regulators of cell cycle, pluripotency, and cell fate transitions in many cell types, especially germ cells. The complex nature of the germ cell life cycle has made it difficult to discern roles for each type of small RNA at every developmental stage. This review has summarized the broad scope of research that has been undertaken to begin to understand the role small RNAs are playing in this very important cell type. Recent technological advances and newer genetic models are paving the way for an even better understanding of how these small molecules regulate significant germ cell fate transitions. What small

RNAs are essential for germline sex-specific development? How might piRNAs direct epigenetic changes, and what is the full mechanism of their biogenesis? How is miRNA activity, but not siRNA activity, suppressed in the vertebrate egg? What are the small RNAs and pathways they regulate that are clinically relevant for GCT development and treatment? Further research will answer these questions and provide insight into how small RNAs shape our germline and, thus, the survival of our species.

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