The Role of *mir-290-295* in Murine Embryonic and Germ Cell Development

by

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Abstract

MicroRNAs, 22nt long RNAs derived from hairpin transcripts, are important regulators of gene expression and have been shown to participate in regulation of every biological process known to date. *Mir-290* through *mir-295* (*mir-290-295*) is a mammalian-specific miRNA cluster that, in the mouse, is expressed specifically in early embryos and embryonic germ cells. This thesis examines the *in vivo* consequences of targeted deletion of *mir-290-295* in the developing mouse embryo. *Mir-290-295* deficiency results in a partially penetrant embryonic lethality. *Mir-290-295* is not required for early preimplantation development but is instead required for later postimplantation development. In surviving *mir-290-295* deficient embryos, female but not male fertility is compromised. This impairment in fertility arises from a defect in migrating primordial germ cells and occurs equally in male and female mutant animals. Male *mir-290-295-/-* mice, due to the extended proliferative lifespan of their germ cells, are able to recover from this initial germ cell loss and are fertile. Female *mir-290-295-/-* mice are unable to recover and are sterile due to premature ovarian failure.

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Chapter 1

Introduction

(Overview of microRNA Function, Early Mammalian Development, Germ-Cell Development and *mir-290-295* Functions in ES cells)

Summary

MicroRNAs are ~22nt long RNAs derived from hairpin transcripts. MicroRNAs (miRNAs) are important regulators of gene expression (Bartel, 2009) and have been shown to participate in regulation of every biological process known to date (Fabian et al., 2010). This thesis will explore the *in vivo* consequences of *mir-290-295* loss in the mouse. Knowledge of miRNAs and the modes in which they function is a prerequisite for understanding the consequences of *mir-290-295 in vivo* deficiency. Because this thesis concerns a mammalian miRNA cluster, I will focus exclusively on animal (and not plant) miRNAs in this introduction. Furthermore, since miR-290 cluster miRNAs are expressed in the early embryo and germ cells, a general understanding of the early embryo and early germ-cell development is required. This chapter is divided into four parts. The first part gives an overview of miRNA function while the second and third parts cover early embryonic development and germ-cell development, respectively. The last part concludes this chapter with a review of the current knowledge of miR-290 family miRNA function.

PART I: Overview of microRNA Biogenesis and Modes of Function

Discovery of microRNAs

The first microRNA, *lin-4*, was uncovered in a screen for heterochronic genes which control the timing and sequence of developmental events in *C. elegans*. The *lin-4 (lf=loss of function)* mutant reiterated early fates inappropriately during late developmental stages resulting in a lack of adult structures (Lee et al., 1993). Oddly, the *lin-4* genomic DNA that rescued this mutant phenotype did not show any conserved open reading frames and all

frameshift and nonsense mutations introduced into the rescue DNA did not affect rescue activity. In addition, comparison of four nematode species revealed high conservation of the *lin-4* gene, but no conserved open reading frame or 3rd position degeneracy. Taken together these results led the Ambros group to conclude that *lin-4* did not encode a protein product but rather two small RNAs – a short 22nt RNA and its longer, 61nt precursor (Lee et al., 1993).

Soon *lin-4* was joined by another small RNA, *let-7* (Reinhart et al., 2000). These small RNAs were originally thought of as mere curiosities of the nematode until Pasquinelli and colleagues made the astounding discovery that *let-7* sequence and temporal expression were conserved throughout all bilaterian animals (Pasquinelli et al., 2000). Spurred on by this discovery, the Ambros, Bartel, and Tuschl groups cloned small RNAs from several animal species (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). All three groups found that these tiny RNAs, which they termed microRNAs (abbreviated as miRNAs), were part of a large class of small RNAs with potential regulatory roles. Further work by the Bartel, Carrington, Chen, and Matzke labs demonstrated that miRNAs were not only found throughout the animal kingdom but were pervasive in the plant kingdom as well (Llave et al., 2002; Mette et al., 2002; Park et al., 2002; Reinhart et al., 2002). These four groups cloned miRNAs from *Arabidopsis* and identified their orthologs in rice, maize and tobacco. It is important to note that the role of small RNAs in regulation of gene expression in plants was already appreciated prior to these cloning experiments of 2002. In 1999, Hamilton and Baulcombe showed that the phenomenon of posttranscriptional gene silencing in plants involved the degradation of homologous mRNA and the production of small RNA species (Hamilton and Baulcombe, 1999). Once further work on RNA-induced gene silencing revealed the significance of the above two characteristics in miRNA-mediated gene regulation, the search for miRNAs in plants began.

Since these original miRNA cloning experiments of 2001 and 2002, the number of miRNAs discovered has greatly expanded and miRNAs have been implicated in almost every biological process known (Bushati and Cohen, 2007; Bartel, 2009; Ghildiyal and Zamore, 2009; Fabian et al., 2010). Currently, the microRNA database, miRbase (Release 17, April 2011) (Griffiths-Jones, 2004; Griffiths-Jones et al., 2006; Griffiths-Jones et al., 2008; Kozomara and Griffiths-Jones, 2011), lists 720 *Mus musculus* miRNAs, 358 *Danio rerio* miRNAs, 238 *Drosophila melanogaster* miRNAs, 207 *Caenorhabditis.elegans* miRNAs, and 1424 *Homo sapiens* miRNAs.

Genomic organization of microRNAs

The distribution of miRNAs throughout the genome is not random (Lagos-Quintana et al., 2001; Lau et al., 2001). Fifty percent of mammalian miRNAs are found in close proximity to other miRNAs (Kim et al., 2009). These clusters are often transcribed as one polycistronic unit (Lee, Y. et al., 2002). Most mammalian miRNA genes have multiple isoforms which are presumed to have arisen by gene duplication (Kim et al., 2009).

MiRNA loci can be found in non-coding transcriptional units as well as protein-coding transcriptional units. In addition, miRNAs can be encoded by the exonic or intronic regions within these units. Approximately 20% of all mammalian miRNAs are derived from the exonic regions of transcriptional units while about 80% of all mammalian miRNAs are derived from the intronic regions of transcriptional units (Rodriguez et al., 2004; Kim and Kim, 2007). About 75% of these intronic, miRNA-encoding regions are located within protein-coding transcriptional units while ~25% are located within non-coding transcriptional units (Rodriguez et al., 2004).

siRNA interlude

At the turn of the last century, miRNAs were not the only small RNAs attracting attention. Advances in understanding the biogenesis of another type of small RNA, short interfering RNA (siRNA), would lead to insights into the mechanism of miRNA generation. In 1998, Fire and Mello showed that double-stranded RNA (dsRNA) could trigger mRNA degradation and that this loss of mRNA would phenocopy the loss-of-function mutation of the corresponding genetic loci (Fire et al., 1998). They termed this phenomenon of dsRNA-triggered, posttranscriptional gene silencing, RNA interference (RNAi). Soon RNAi was found to be mediated by small ~ 22nt RNAs generated from exogenous dsRNA by the RNAseIII enzyme Dicer (Hamilton and Baulcombe, 1999; Zamore et al., 2000; Elbashir et al., 2001). To affect silencing, one strand of the ~ 22nt dsRNA would incorporate into the RNA-induced silencing complex (RISC) and direct cleavage of its perfectly complementary target mRNA (Tuschl et al., 1999; Hammond et al., 2000; Bernstein et al., 2001). The similar 22nt size of the siRNA and the known *let-7* and *lin-4* miRNAs raised the intriguing possibility that these pathways shared common biogenesis machinery. In 2001, three groups showed that miRNAs were also cleaved by Dicer and incorporated into RISC to induce silencing (Grishok et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001).

Although there are similarities between siRNA and miRNA biogenesis there are also differences. The establishment of an *in vitro* processing assay helped to determine the initial steps in miRNA biogenesis (Lee, Y. et al., 2002) which will be detailed below and which differ substantially from the initial steps in siRNA biogenesis. While miRNAs are processed from hairpin precursors, siRNAs are derived from either much longer RNA hairpins or bimolecular RNA duplexes (Bartel, 2009).

Biogenesis of canonical microRNAs

With few exceptions, all miRNA genes are transcribed as long primary transcripts by RNA polymerase II (Lee et al., 2004; Borchert et al., 2006). Primary canonical miRNA transcripts (pri-miRNAs) are first processed into 70nt fragments in the nucleus by the Microprocessor complex, consisting of DGCR8 (Drosha-DiGeorge syndrome critical region 8) and the RNAseIII enzyme Drosha (Lee et al., 2003; Han et al., 2004; Han et al., 2006; Yeom et al., 2006). (Fig. 1.1)

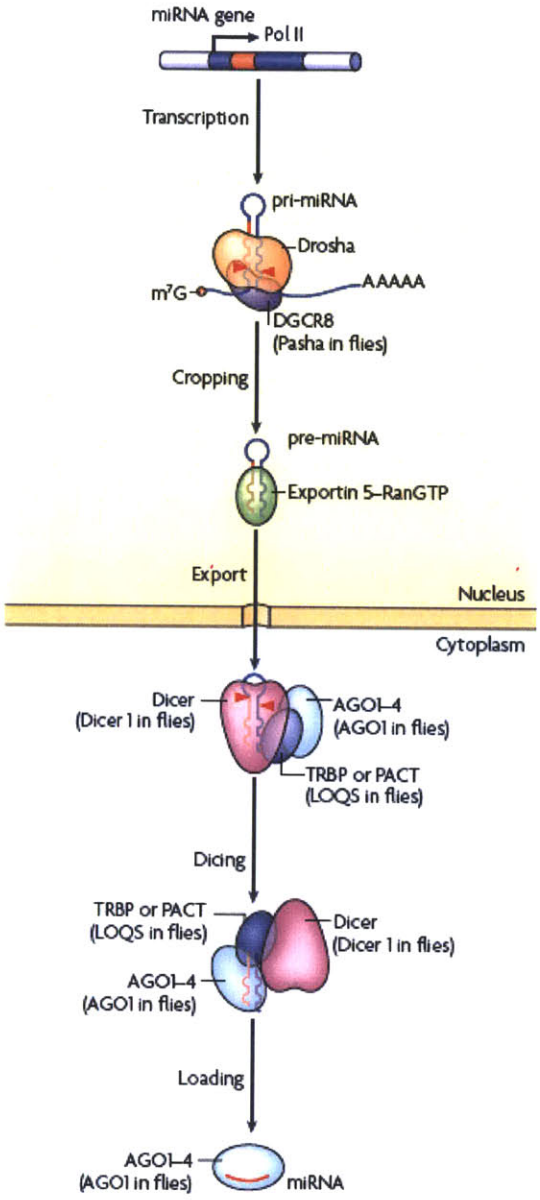

Figure 1.1: Biogenesis of canonical microRNAs. Taken from Kim, V.N. 2009.

The 70nt, hairpin-shaped pre-miRNA is then transported from the nucleus in a Ran-GTP-mediated manner by exportin 5 (EXP5) which recognizes the 2nt short 3'overhang (Zamore et al., 2000; Lee, Y. et al., 2002; Yi et al., 2003; Bohnsack et al., 2004; Lund et al., 2004).

In the cytoplasm, the RNAseIII enzyme Dicer cleaves the 70nt hairpin into 22nt miRNA duplexes (Bernstein et al., 2001; Grishok et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001; Knight and Bass, 2001). Human Dicer associates with double-strand RNA binding proteins, TRBP (TAR RNA binding protein) and PACT (also known as PRKRA) (Chendrimada et al., 2005; Haase et al., 2005; Lee et al., 2006). The exact biochemical function of these double-strand RNA binding proteins is unknown although they are presumed to contribute to the formation of the RNA-induced-silencing complex (RISC) (Kim et al., 2009).

After Dicer cleavage, one strand of the miRNA duplex (the guide strand, miRNA) is loaded into the Argonaute (AGO) -containing RISC while the other strand (miRNA*) is degraded (Hutvagner and Zamore, 2002; Mourelatos et al., 2002). It is not known how one strand of the duplex is chosen over the other although studies with siRNAs suggest that the thermodynamic properties of the strands are a critical determinant (Khvorova et al., 2003; Czech and Hannon, 2011). Some hairpins yield miRNAs from both strands of the duplex which are found at relatively equal concentrations in the cell. In these cases, the miRNA/miRNA* nomenclature is not used and the two miRNAs from the duplex are termed miR-5p (5'arm) and miR-3p (3'arm) (Griffiths-Jones et al., 2006; Kim et al., 2009).

Once the RISC has assembled, the guide miRNA then directs the RISC to sites in the target mRNA that are often, but not exclusively, located in the 3'UTR (Lai, 2002). Perfect base pairing between the miRNA and its target results in AGO2-mediated cleavage of the mRNA (Hutvagner and Zamore, 2002; Liu et al., 2004; Song et al., 2004). Although there are some examples of metazoan, miRNA -directed mRNA cleavage, most animal microRNAs pair imperfectly to their mRNA targets (Yekta et al., 2004; Bartel, 2009). In fact, in animals recognition between the guide miRNA and its target mRNA is mediated mainly by nucleotides 2-7 at the 5' end of the miRNA (Lewis et al., 2003; Brennecke et al., 2005; Krek

et al., 2005). These 6 nucleotides in the miRNA are known as the seed sequence and are most often exactly complementary to the target sites in the mRNA. Even though metazoan miRNAs do not usually direct target cleavage, most miRNAs still mediate mRNA degradation (Bagga et al., 2005; Lim et al., 2005; Guo et al., 2010). This is accomplished through miRNA-mediated mRNA destabilization, indirectly through miRNA-mediated translational repression, or a combination of both (Bartel, 2009; Fabian et al., 2010).

Non-canonical microRNAs

The above section outlines the biogenesis of canonical miRNAs. However, miRNAs are also generated by other RNA metabolic pathways, almost all of which are Microprocessor-independent but Dicer-dependent. For example, some non-canonical miRNAs, known as mirtrons, are short intronic transcripts that are processed by the splicing machinery to yield pre-miRNA like hairpins. Mirtrons bypass the DGCR8 and Drosha processing step and merge with the traditional microRNA biogenesis pathway upon nuclear export (Berezikov et al., 2007; Okamura et al., 2007; Ruby et al., 2007). Another alternative miRNA processing pathway circumvents the Drosha/DCGR8 step by utilizing Dicer to generate both precursor and mature miRNAs (Ender et al., 2008; Glazov et al., 2009; Taft et al., 2009; Brameier et al., 2011).

Because all of the alternative pathways converge at the Dicer processing step, Dicer was believed to be absolutely required for all miRNA (and siRNA) production. Recently one exception to this rule was discovered. The locus encoding miR-451 is transcribed, processed to pre-miRNAs by the Microprocessor complex, exported, and directly cleaved by AGO2, thereby bypassing Dicer (Cheloufi et al., 2010; Cifuentes et al., 2010; Yang et al., 2010).

Biological functions of microRNAs

Introduction to microRNA modes of regulation

What are the specific regulatory roles of microRNAs? Given that miRNAs have been shown to be involved in almost every biological process to date (Fabian et al., 2010), it is unlikely that each miRNA will function identically or even that one miRNA will function identically in all contexts. However, this does not mean that there are not common modes by which miRNAs can act (Flynt and Lai, 2008). These modes can be defined based on whether one focuses on a specific miRNA-target interaction or whether one considers the overall function of a certain miRNA within a cell. If one considers a particular miRNA-target interaction, parameters such as degree of miRNA-mediated target repression and the overall biological significance of this specific repression can be used to classify miRNA function. For example, does the given microRNA act as a switch to turn off expression of its target or act as a rheostat to tune expression of its target? Is there any biological significance to this repression? Because most miRNAs target many different messages one can consider the overall function of the miRNA with respect to its many different targets. Does loss of this microRNA (and all its mRNA-target interactions) lead to an overt biological phenotype or is the phenotype more subtle or is there no phenotype at all?

Original paradigm: microRNAs function to throw a developmental switch

The original paradigm for microRNA function, based on insights from the first miRNA mutants, suggested that miRNAs function as switches to turn off expression of a few target genes and that loss of these targeting interactions leads to strong phenotypes. The first clue as to the function of miRNAs came from another mutant isolated from the original screen that

identified *lin-4* (Lee et al., 1993). The *lin-14 (lf= loss-of-function)* nematodes precociously executed later developmental stage programs during early stages. This phenotype was opposite to the reiteration of early developmental stages observed in *lin-4(lf)* mutants and further genetic experiments indentified *lin-4* as a negative regulator of *lin-14*. The model by which *lin-4* miRNA decreased lin-14 protein levels was rooted in the observation that the *lin-14* 3'UTR contained 7 copies of a 14-19bp sequence complementary to a portion of the *lin-4* sequence. Additional experimentation proved that the 3'UTR of *lin-14* was necessary and sufficient for *lin-4*-mediated posttranscriptional regulation (Wightman et al., 1991). Therefore the *lin-4* miRNA promotes the transition to the second larval (L2) stage by binding to the 3'UTR of *lin-14* and thereby negatively regulates *lin-14* expression (Arasu et al., 1991; Wightman et al., 1993).

Similarly, *let-7* was found to target *lin-41*. Interestingly, the phenotype of *let-7(lf)* mutants could be mostly suppressed by decreasing the activity of *lin-41*, suggesting that misregulation of one *let-7* target is primarily responsible for the *let-7(lf)* phenotype (Reinhart et al., 2000; Grosshans et al., 2005). In both the *lin-4* and *let-7* examples, loss of a specific miRNA caused misregulation of a few target genes resulting in bold, easily observable phenotypes. Furthermore, in both these examples the expression of the miRNA and its target were mutually exclusive because expression of the miRNA reduced the level of its target(s) to inconsequential levels.

MicroRNAs can tune expression of target genes

The original paradigm for miRNA function does not hold true for all miRNAs. *Lin-4(lf)* and *let-7(lf)* mutants are not typical of most miRNA loss-of-function mutants. Perhaps this is not surprising considering that the *let-7* and *lin-4* mutants were isolated in genetic screens which, by their very nature, would favor the isolation of mutants with strong phenotypes.

Not every miRNA completely abolishes expression of its target gene(s). A miRNA can also "tune" the expression level of a target RNA (Bartel and Chen, 2004). In contrast to the "general switch" miRNA-target interaction illustrated by *lin-4* - *lin-14* and *let-7* - *lin-41*, in the "tuning" miRNA-target interaction the miRNA does not eliminate target gene activity. This tuning mode of action can be used to set thresholds or reduce the effect of noise on a system. Tuning interactions are not merely theoretical, they can be experimentally demonstrated *in vivo*.

A classic example of a tuning target interaction is between the conserved miR-8 and its target *atrophin* in *Drosophila* (Karres et al., 2007). *MiR-8 (lf)* mutants showed various morphological and behavioral abnormalities triggered by increased apoptosis in the central nervous system. *Atrophin* was found to be a direct functional target of miR-8. Surprisingly, many of the mutant phenotypes could be ameliorated by removing 1 copy of the *atrophin* gene in the *miR-8(lf)* background, suggesting that the major role of miR-8 was to regulate *atrophin*. Selective elimination of *atrophin* in miR-8 expressing cells caused defects that mirrored known *atrophin* loss of function phenotypes. This suggested that both *atrophin* and miR-8 expression were required *in the same cell* for proper cellular function. Taken together these results showed miR-8 tunes *atrophin* levels to an optimal level.

The example of miR-8 also illustrates the importance of cellular context in miRNA – target relationships. Interestingly, *atrophin* is not the biologically relevant target of miR-8 in every *Drosophila* tissue. In *Drosophila* fat body cells miR-8 directly represses *USH* thereby activating insulin signaling and ultimately controlling body size (Hyun et al., 2009).

MicroRNAs target multiple mRNAs

These above examples would suggest that miRNAs only target a small handful of genes. However, work on miRNA target prediction gave evidence that miRNAs can target hundreds

of genes (Bartel, 2009). While for some miRNAs, such as *lin-4* and *let-7*, genetic analysis can suggest targets, for most miRNAs, little is known initially, and computer algorithms must be used to predict potential targets. Residues 2-7 of the miRNA, known as the seed, typically effect repression by binding to 3'UTR elements in the target mRNA (Lai, 2002). The computational programs make use of this complementary pairing to search for potential target mRNAs (Lewis et al., 2003; Lall et al., 2006; Betel et al., 2008; Bartel, 2009; Friedman et al., 2009). Computer algorithms further rely, to varying degrees, on target-site conservation among species as well as additional local sequence features in the miRNA and its target in order to improve prediction accuracy (Grimson et al., 2007; Bartel, 2009).

These computational algorithms suggested that each miRNA has many conserved targets (Brennecke et al., 2005; Krek et al., 2005; Lewis et al., 2005; Stark et al., 2005). They predicted that each vertebrate miRNA had greater than 100 targets and that over a third of human genes were predicted to be targeted by miRNAs. Additional analysis showed that more than half of mammalian mRNAs are under selective pressure to retain pairing to microRNAs (Friedman et al., 2009). Experimental analysis supported these computational predictions. Ectopic expression of miR-124 in HeLa cells caused the expression profile to switch toward that of the brain, the organ in which miR-124 is preferentially expressed (Lim et al., 2005). Ectopic expression of miR-1 caused the expression profile to switch toward that of muscle, the tissue in which miR-1 is preferentially expressed (Lim et al., 2005). These data propose a model whereby tissue-specific miRNAs regulate large numbers of transcripts within a cell to help shape tissue identity.

Proteomics studies also showed that a single miRNA can translationally repress hundreds of direct target genes (Baek et al., 2008; Selbach et al., 2008). Interestingly the actual effect on mRNA level and protein output was relatively mild, rarely greater than fourfold, indicating that rather than acting to completely abolish target gene expression, miRNAs act as a

rheostat to tune target expression to an appropriate level. These large-scale genomics and proteomics studies relied mostly on *in vitro* cell culture experiments. What do *in vivo* studies reveal about the number of targets regulated by a miRNA?

One microRNA can have many *in vivo* targets: miR-430 and the maternal-zygotic transition

An elegant *in vivo* example of a miRNA that controls multiple targets is zebrafish miR-430. Giraldez and colleagues showed that miR-430 targets hundreds of maternal transcripts for deadenylation and subsequent degradation, thereby helping to clear maternal transcripts during the maternal-to-zygotic transition (Giraldez et al., 2006). Based on this information, in this context, miR-430 - target interactions can be characterized as general switch-target interactions because miR-430 eliminates expression of targeted maternal transcripts. Interestingly, even in the absence of miR-430, when maternal transcripts are not efficiently cleared, the zebrafish embryo can still activate the zygotic program. With respect to the overall function of a miRNA, this suggests a different category of miRNA function than that seen with *lin-4(lf)* and *let-7(lf) C. elegans* mutants. Unlike the *lin-4(lf)* and *let-7(lf)* mutants, zebrafish mutants lacking miR-430 could still transition from one stage (maternally-controlled state) to the next (zygotically-controlled state). MiR-430, rather than inducing a developmental transition from one state to the next, helped to sharpen this transition. Although the relationships between miR-430 and its maternal transcript targets can be defined as general switch interactions, the overall function of miR-430 is not to throw a developmental switch from one state to the next. In the absence of miR-430 the switch from maternal control to zygotic control still occurs. Thus, the miR-430 data support the view that some miRNAs can fine tune development to ensure robustness (Giraldez et al., 2006).

Not all targets are always equal

The above example of miR-430 illustrates that *in vivo* one miRNA can regulate hundreds of targets. As previously mentioned, most miRNAs are predicted to have hundreds of targets and therefore, like miR-430, may actually regulate most of these targets *in vivo*. But are all of these predicted miRNA-target interactions biologically relevant in a given context? Is each miRNA-target interaction of equal biological significance or are some miRNA-target interactions more crucial than others?

Studies of mice deficient for specific miRNAs provide *in vivo* evidence that, at least in some cases, misregulation of one target can often explain substantial aspects of the knockout phenotype. For example, defects conferred by loss of miR-150 in mice can be mostly attributed to overexpression of its target *c-myb* (Xiao et al., 2007). *Mir-150* homozgyous knockout mice are viable and fertile but display a block in immune system development at the pro to pre-B-cell transition. Although many mRNAs have miR-150 binding sites, out of several potential targets tested only C-MYB was affected by changes in miR-150 levels. Impressively, only a 30-35% reduction in C-MYB protein levels was needed to recapitulate the phenotype conferred by ectopic expression of *mir-150*. In addition to showing that one miRNA-target interaction may be more significant than others, the miR-150 example demonstrates how small changes in protein levels of a target can yield a significant phenotype.

MiR-223 is essential for the normal neutrophil maturation in the mouse (Johnnidis et al., 2008). Proteomics studies on wild-type and *miR-223*-deficient neutrophils indicated that miR-223 has more than 200 targets (Baek et al., 2008). However, genetic ablation of only one target, the transcription factor *Mef2c,* corrected the neutrophil defect in *miR-223*-deficient mice (Johnnidis et al., 2008).

The cleanest way to determine the *in vivo* functional significance of one miRNA-target interaction would be to generate mice with a mutation in the microRNA binding site of the target mRNA. This type of experiment was done with the miR-155 target *AID* (activation induced cytidine deaminase) and revealed that miR-155-mediated repression of *AID* helped prevent a potentially oncogenic translocation (Dorsett et al., 2008). Sometimes nature has already done this type of experiment. A point substitution creating an illegitimate single miR-1 regulatory site in the sheep *Myostatin* 3'UTR leads to muscular hypertrophy in Texel sheep (Clop et al., 2006).

Conclusions about miRNA functions

As all of the experiments included in Part I have shown, there are many modes in which miRNAs can function. A miRNA can completely abolish expression of its target gene or tune expression of its target gene to an optimal level. MiRNAs can have many targets *in vivo* yet sometimes only a few of these miRNA-target interactions have bold biological consequences in a given context. These different examples demonstrate that not all miRNAs act the same. Even the same miRNA can act differently in different contexts. Therefore when studying the roles of *mir-290-295* in the mouse it will be important to look closely at the resulting phenotypes and understand that the explanation behind them is not likely to be as simple as miR-290 microRNAs targeting a few mRNAs to throw a developmental switch.

PART II: Role of microRNAs in Early Mammalian Development

Introduction to early mammalian development

After the sperm and oocyte fuse and the chromosomes of the two gametes align, the zygote undergoes its first mitosis, producing a two-cell embryo (Arnold and Robertson, 2009). The

eight blastomeres produced during the first three cell divisions of the embryo are all considered equivalent (Suwinska et al., 2008; Tarkowski et al., 2010). After the eight-cell stage, when the embryo undergoes compaction and forms the 16-cell stage morula, the first lineage allocation occurs (Sutherland et al., 1990). The small inner cells of the morula comprise the inner cell mass (ICM) while the outer polarized cells comprise the trophectoderm (TE). These lineage decisions are mediated by the transcription factors TEAD4 and CDX2 in the trophectoderm and OCT4 in the inner cell mass (Arnold and Robertson, 2009). Even though there are two distinct populations of cells at the morula stage, cells of the 16-cell stage embryo retain the capacity to produce all developmental lineages; blastomeres lose totipotency only after the 32-cell stage (Johnson and McConnell, 2004; Suwinska et al., 2008; Tarkowski et al., 2010) (Fig.1.2).

By 3.5 days after fertilization (E3.5), the morula has become a blastocyst which is characterized by a central fluid-filled cavity called the blastocoel.. At the late blastocyst stage the embryo is composed of three distinct lineages (Arnold and Robertson, 2009). The outer trophectodermal layer, specified at the morula stage, directs implantation and expands to form the progenitors of the placenta, specifically the extraembryonic ectoderm (ExE) and the ectoplacental cone (EPC). The outermost layer of cells that comprised the ICM has given rise to the primitive endoderm (PE).

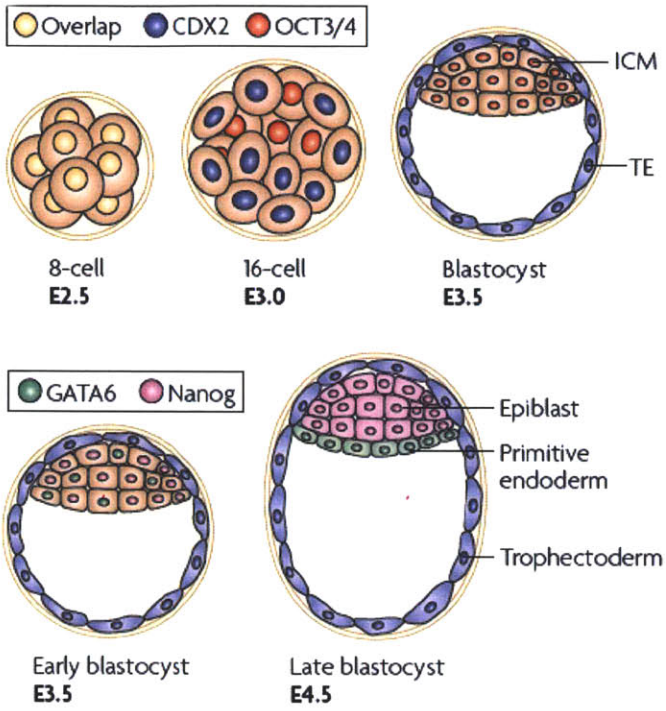

Figure 1.2. Lineage segragation in the blastocyst. Taken from Arnold 2009.

The PE is originally characterized by expression of *GATA6* and will give rise to both the parietal and visceral endoderm (Arnold and Robertson, 2009). The parietal endoderm migrates to the surface of the embryo and contacts the maternal tissue while the visceral endoderm remains in contact with the embryo, surrounding the extraembryonic ectoderm and epiblast. Visceral endoderm will give rise to the endodermal layer of the visceral yolk sac and plays an important role in patterning the early embryo. The third distinct lineage of the late blastocyst stage is the epiblast which arose from *Nanog* expressing ICM cells and will give rise to all germ layers of the embryo and some extraembryonic mesoderm.

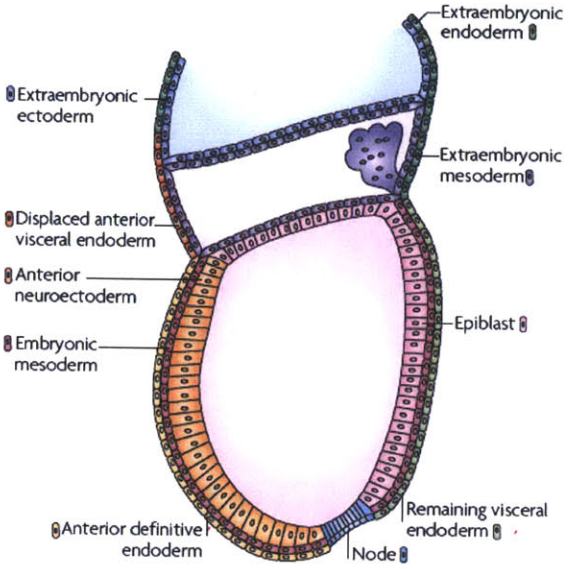

Figure 1.3. Formation of different cell types at gastrulation. Taken from Arnold 2009.

Implantation of the embryo into the uterine wall begins around E5.0. At E6.0, the epiblast cells converge to the posterior proximal region of the epiblast to form the primitive streak. By E6.5 gastrulation has begun and over the next 36 hours the primitive streak will elongate toward the distal tip of the embryo (Arnold and Robertson, 2009) (Fig.1.3). Epiblast cells entering the primitive streak at the posterior and intermediate regions of the embryo will become extraembryonic mesoderm and nascent embryonic mesoderm, respectively. Epiblast cells entering the primitive streak at the anterior end will give rise to definitive endoderm. Epiblast cells that do not ingress through the primitive streak will give rise to neuroectoderm .

Role of microRNAs in early development

Maternal microRNAs were originally believed to be essential for mouse zygotic development because embryos from *Dicer-/-* oocytes, fertilized with wild-type sperm, failed to proceed

through the first cell division (Murchison et al., 2007; Tang et al., 2007). However maternal and zygotic *Dgcr8*-deficient embryos were morphologically normal at E3.5, suggesting that the maternal *Dicer-/-* phenotype was not due to loss of miRNAs (Suh et al., 2010). This and other recent observations have suggested that miRNA function is suppressed in mouse oocytes and early preimplantation embryos (Ma et al., 2010; Ohnishi et al., 2010). Instead, endogenous siRNAs, whose biogenesis requires Dicer, play the main role in early preimplantation development (Ohnishi et al., 2010; Suh et al., 2010).

However, a role for maternally-deposited miRNAs later on in development has not been excluded. Litter sizes from females with *Dgcr8-/-* oocytes, fertilized by wild-type males, are smaller than expected (Suh et al., 2010). Since early preimplantation development does not appear to be affected by loss of maternal miRNAs, maternal miRNAs likely play a role in peri/early postimplantation development (Suh and Blelloch, 2011).

Zygotic deletion of *Dicer, Dgcr8*, or *Ago2* results in early postimplantation lethality around E7.0 in the mouse (Bernstein et al., 2003; Liu et al., 2004; Morita et al., 2007; Wang et al., 2007). Although *Dgcr8-/-* embryos would provide the cleanest model for examining the effects of miRNA loss on early development, *Dicer-/-* embryos have been more extensively studied. *Dicer* homozygous knockout embryos initiate gastrulation but show a failure to elongate the primitive streak and also display defects in early embryonic patterning of the epiblast (Bernstein et al., 2003; Spruce et al., 2010). Additional work showed that *Dicer-/-* embryos have defects in all three cell types of the early implanted embryo – primitive endoderm, epiblast and trophectoderm (Spruce et al., 2010). *Dicer-/-* epiblasts displayed increased apoptosis which was presumed to result from the loss of miR-17-92 and miR-106b which both target the proapoptotic protein Bim. However, no rescue experiments were performed to directly test this hypothesis. *Dicer-/-* primitive endoderm cells showed reduced proliferation and increased differentiation. This was attributed to loss of miRNA-mediated

downregulation of Mapk inhibitors. *Dicer-/-* trophectodermal cells showed an inability to maintain the trophoblast stem cell compartment, presumably due to loss of miRNA-mediated repression of cell cycle inhibitors p21 and p57. Further experiments will be required to test these hypotheses.

Part III: Germ-Cell Development

Germ-cell specification

In multicellular organisms the formation of the germ-cell lineage can follow one of two modes– preformation or epigenesis (Extavour and Akam, 2003). In model organisms such as *D. melanogaster* and *C.elegans* the germ-cell lineage is preformed by cytoplasmic maternal determinants set aside in the egg. In the mouse and other mammals, germ-cell formation proceeds via epigenesis.

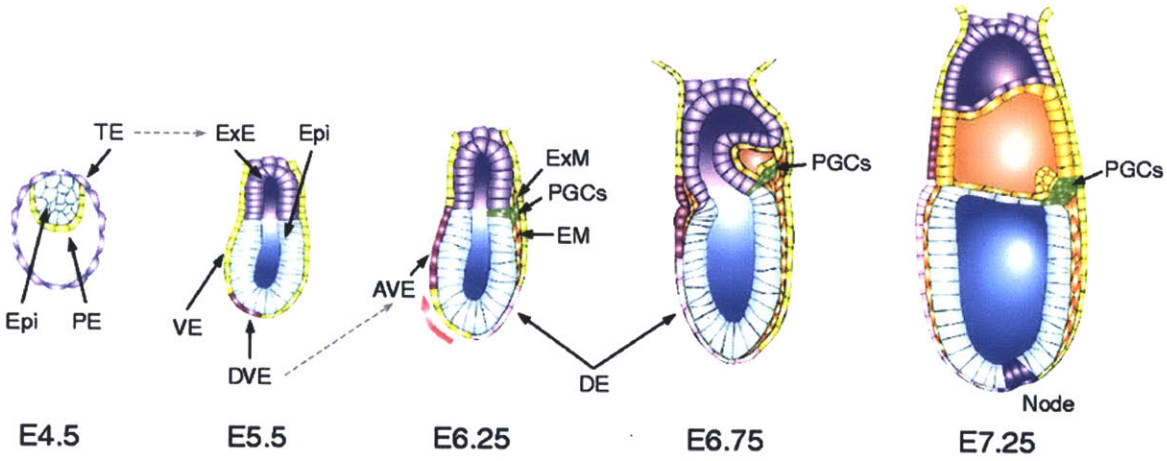

Figure 1.4: Schematic representation of the emergence of PGCs in the mouse embryo. Taken from Saitou 2010. Abbreviations are as follows: Epi = Epiblast, PE= primitive endoderm, TE= trophectoderm, ExE=extraembryonic ectoderm, VE=visceral endoderm, DVE=distal visceral endoderm, AVE= anterior visceral endoderm, ExM= extraembryonic mesoderm, EM=embryonic mesoderm, DE=definitive endoderm. (In mice the distal visceral endoderm translocates to the future anterior end of the embryo, thereby helping to set up the anterior-posterior axis. The AVE is a signaling center that helps to pattern the embryo.)

In the mouse, between E5.0 and E6.25, nascent primordial germ cells (PGCs) are induced in the proximal epiblast by Bmp4 signals emanating from the extraembryonic ectoderm (Lawson and Hage, 1994; Ohinata et al., 2009) (Fig. 1.4). These signals are transduced by Smad1 (and 5) and Alk3 (and Alk6) in the epiblast. Other signaling molecules cooperate with Bmp4 in order to ensure that the primordial germ cells are only formed in the region of the (proximal) epiblast that experiences the highest concentration of Bmp4. For example, Bmp8b blocks anterior visceral endoderm (AVE) development, thereby preventing the inhibitory signals produced by the AVE from affecting germ-cell specification. Bmp2, very similar in structure to Bmp4, is produced in the visceral endoderm, and is hypothesized to provide additional signal to ensure sufficient Bmp levels for germ-cell specification. Wnt3 ensures that the epiblast cells are competent to respond to the Bmp signals from E5.5 to E6.5, although the mechanism by which this happens is unclear (Ohinata et al., 2009).

Bmp4 signaling leads, via unknown mechanisms, to expression of *Blimp1* (B-lymphocyte induced maturation protein 1; also known as *Prdm1*, PR-domain-containing protein 1) and *Prdm14* (PR-domain-containing protein 14) in the proximal epiblast cells destined to become PGCs. This group of *Blimp1- Prdm14* double-positive cells increases in number, gains *Dppa3* (developmental pluripotency associated gene 3, also known as *stella* or *Pgc7*) expression, and around E7.5 forms a cluster of 40 TNAP (tissue non-specific alkaline phosphatase)-positive cells at the base of the allantois. *Dppa3* (Saitou et al., 2002; Sato et al., 2002; Payer et al., 2003) and TNAP (Chiquoine, 1954) are very useful markers for the germ-cell lineage, even though they are not required for germ-cell development (MacGregor et al., 1995; Payer et al., 2003; Bortvin et al., 2004)

Blimp1 and *Prdm14* are the main players in the emergence of the germ-cell lineage. *Blimp1-/-* mice display a small number of PGC-like cells that neither migrate properly nor

proliferate (Ohinata et al., 2005; Vincent et al., 2005). Interestingly, even *Blimp1 +/-* mice display reduced numbers of PGCs. *Prdm14-/-* mice also have a few PGC-like cells that fail to be maintained (Yamaji et al., 2008).

Single-cell-expression profiling of PGC precursors and their somatic neighbors in wild-type and *Blimp1-/-* embryos has helped to elucidate the three main events required for germ-cell specification – re-expression of pluripotency-associated genes, repression of the somatic program, and epigenetic reprogramming (Yabuta et al., 2006; Kurimoto et al., 2008b). Around the time of PGC specification, cells of the epiblast are under mesoderm-inducing signals which promote downregulation of pluripotency-associated genes (such as *Sox2*) and upregulation of mesodermal-patterning genes (such as *Hoxb1*). Successful germ-cell specification therefore requires PGC precursors to upregulate pluripotency-associated genes and downregulate expression of somatic genes. These somatic genes include S-phase-promoting genes, growth factor genes, and mesodermal-patterning genes. In addition, the *de novo* DNA methyltransferases (*Dntm3a* and *Dnmt3b*) are also repressed in the nascent PGCs. In contrast, the genes upregulated in *Blimp1*-positive PGC precursors are not only pluripotency-associated genes such as *Sox2* and *Nanog* but also germ-cell specific genes. In *Blimp1-/-* embryos, the PGG-like cells express similar levels of somatic genes as their somatic neighbors (Ohinata et al., 2005; Kurimoto et al., 2008b; Kurimoto et al., 2008a). Furthermore, the *Blimp1-/-* early PGCs fail to upregulate pluripotency-associated genes and germ-cell specific genes.

In *Prdm14-/-* embryos, the PGC-like cells are able to repress the somatic program but unable to upregulate germ-cell specific and pluripotency-associated genes. In addition, *Prdm14-/-* PGC precursors cannot generate pluripotent embryonic germ (EG) cells *in vitro* (Yamaji et al., 2008). This suggests that *Blimp1* is the dominant factor in suppressing the

somatic program while both *Blimp1* and *Prdm14* are required for upregulation of pluripotency-associated and germ-cell specific genes.

Both *Blimp1* and *Prdm14* are also necessary for the epigenetic reprogramming that occurs in the germ-cell lineage (Seki et al., 2005; Seki et al., 2007; Kurimoto et al., 2008a). At around E7.25, PGCs show levels of H3K9me2, H3K27me3, and DNA methylation equivalent to those of their somatic neighbors. By E7.5, however, PGCs exhibit decreasing levels of H3K9me2, a repressive modification with high stability. Around E8.0, levels of DNA methylation begin to decrease. Around E8.5, the levels of H3K27me3, a repressive mark with more plasticity, begin to increase. Due to these changes in the epigenetic landscape, between E7.5 and E8.25 the genome of PGCs is relatively free of repressive marks. Interestingly, between E7.5 and E8.25, RNA polymerase II activity is repressed as determined by lack of BrdU incorporation and loss of RNA polymerase II C-terminal-domain phosphorylation. The current idea is that *Blimp1* and *Prdm14* enter into this whole process via their roles in repressing GLP, an enzyme essential for dimethylation of H3K9. By repressing GLP, *Blimp1* and *Prdm14* promote a less severely repressive epigenetic landscape. The reasons for establishing this less repressive nuclear environment are not entirely clear.

The mechanism by which *Blimp1* and *Prdm14* accomplish their tasks is not known. Although BLIMP1 has a histone methyltransferase motif, no enzymatic activity has so far been observed. While BLIMP1 does bind to PRMT5, a histone arginine methyltransferase, the exact significance of this interaction as it relates to germ cell specification is unknown (Ancelin et al., 2006).

Recent experiments studying PGC formation *in vitro* have unearthed a potential supporting role for miRNAs in germ-cell specification. Knockdown of *Lin28* was found to drastically reduce the number of PGCs formed *in vitro* (West et al., 2009). *Lin28* has been

shown to inhibit the formation of mature *let-7* by binding to *let-7* pre-miRNAs and pri-miRNAs and altering their processing or stability (Heo et al., 2008; Newman et al., 2008; Rybak et al., 2008; Viswanathan et al., 2008). In this PGC *in vitro* system, *Lin28* knockdown was associated with upregulation of *let-7* miRNAs and decrease of *Blimp1* expression in PGC precursors. In addition, ectopic *Blimp1* expression was able to rescue the effect of *Lin28* deficiency. Taken together these data suggest that miRNAs might influence PGC specification by regulating *Blimp1* expression

Germ-cell migration

While specification of the germ cells begins at the base of the allantois, the eventual maturation and production of functional gametes will occur in the gonad. Therefore, PGCs must migrate through the developing embryo to the gonad (Fig. 1.5). The generation of a modified *Oct-4*-driven-GFP reporter which allows for live-imaging of PGCs has proved invaluable in studying germ-cell migration (Boiani et al., 2004). The migration of primordial germ cells can be divided into six phases (1) invasion of endoderm (2) incorporation into hindgut (3) random migration within hindgut (4) migration out of the hindgut and into the genital ridges (5) clustering at the ridges (6) death of germ cells in ectopic locations (Molyneaux and Wylie, 2004; Kunwar et al., 2006).

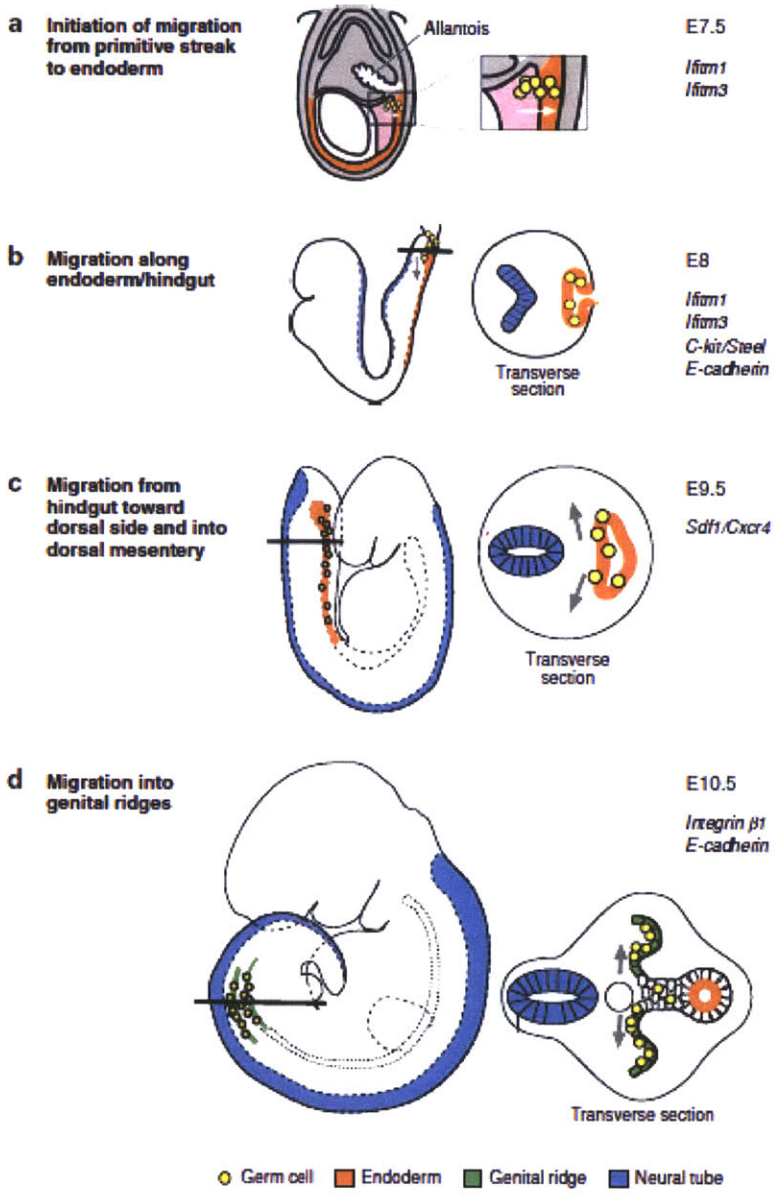

Figure 1.5: Stages of germ cell migration in the mouse. Taken from Kunwar 2006.

Immediately prior to migration into the endoderm, PGC morphology changes from being round to being more polarized (Molyneaux and Wylie, 2004). Furthermore, time-lapse

movies show PGCs of this stage extending cytoplasmic processes, indicating that they are motile (Anderson et al., 2000). After moving through the primitive streak, most PGCs invade the endoderm while some invade the allantois or extraembryonic endoderm. Because cell movement in the allantois is proximal to distal, it is unlikely these PGCs moving to the allantois or the extraembryonic endoderm would be able to migrate against this morphogenetic movement into the hindgut (Downs and Harmann, 1997; Anderson et al., 2000). The mechanisms behind the initiation of PGC migration in the mouse are not well understood. Originally, *Ifitm1* (Interferon-inducible transmembrane protein 1, also known as *fragilis*) was thought to play a role in the movement of PGCs from the epiblast into the endoderm (Tanaka et al., 2005). However, an *Ifitm*-deficient mouse showed no defects in germ-cell migration (Lange et al., 2008).

After PGCs have migrated into the endoderm they are incorporated into the hindgut. The exact mechanism for this is unknown although evidence suggests that passive movement of the soma, rather than active migration of PGCs, might be the major process by which PGCs enter the hindgut. First, at E8.5, PGCs located on the lip of the hindgut pocket have a non-motile morphology (Molyneaux and Wylie, 2004). Second, defects in hindgut expansion can lead to mislocalization of PGCS outside the hindgut. In *Sox17* mutants, the entrance to the hindgut is blocked by defective definitive endoderm cells that are unable to execute the proper morphogenetic movements (Hara et al., 2009). As a result, the majority of PGCs in these mutant animals migrate around the blocked hindgut entrance into the yolk sac. These ectopic PGCs are ultimately unable to reach the genital ridge and presumably die by apoptosis.

PGCs that enter the hindgut are extremely motile although the movements they exhibit are random (Molyneaux et al., 2001). Both *E-cadherin* and the receptor-ligand pair *c-kit /Steel Factor* are thought to play a role in PGC movement in the hindgut (Molyneaux and

Wylie, 2004). Cells of the hindgut epithelium express *E-cadherin* while PGCs at this stage do not; PGCs only upregulate *E-cadherin* upon emigrating from the hindgut (Bendel-Stenzel et al., 2000). This lack of adhesive interaction is presumed to allow for the increased motility of hindgut PGCs.

C-kit and its ligand *Steel Factor* are the two factors most closely associated with PGC motility, survival, and migration. Loss of *Steel Factor* leads to a reduction in PGC numbers by E9.5 (Besmer et al., 1993). PGCs in the W^e/W^e (white extreme allele of *c-kit*) embryos have defects in motility while in the hindgut (Buehr et al., 1993). Furthermore, many PGCs in this mutant are mislocalized to the allantois and the vitelline artery.

Between E9 and E9.5, PGCS emerge from the hindgut and invade the dorsal body wall. The population of PGCs splits into two streams as the germ cells colonize the genital ridges (Molyneaux and Wylie, 2004). Evidence from both *in vivo* and *in vitro* experiments suggests that the ligand *SDF-1* and its receptor *CXCR4* are required for migration from the hindgut to the genital ridges (Ara et al., 2003; Molyneaux et al., 2003). *CXCR4* is expressed by migrating germ cells. *SDF-1* is produced by the genital ridges, however it is also broadly expressed throughout the embryo. Therefore, the mechanism by which *SDF-1* is able to guide PGC migration only to the genital ridges is not clear.

Study of factors involved solely in germ-cell migration is made challenging by the fact that PGCs that mismigrate soon die by apoptosis. During germ-cell migration, apoptosis occurs via both Bax-dependent and Bax-independent mechanisms (Stallock et al., 2003). It is difficult to separate genes that are required for directing the migration of PGCs from genes that are involved in PGC survival.

Around E10.5-E11.0 while most germ cells have colonized the genital ridge, some have not yet reached the gonad and are still located at the midline. Donwregulation of *Steel*

Factor in the midline after E10.5 leads to apoptosis of midline PGCs (Stallock et al., 2003; Runyan et al., 2006).

Gonadal homecoming

After entering the gonad, the germ cells, now termed gonocytes, lose their ability to migrate and downregulate expression of pluripotency-associated genes. The gonocytes continue to proliferate and female germ cells become localized in clusters called cysts while male germ cells become encapsulated by the future seminiferous tubules (Pepling and Spradling, 1998).

Concomitant with their arrival in the gonad, gonocytes show a drastic decrease in levels of DNA methylation (Szabo and Mann, 1995; Walsh et al., 1998; Hajkova et al., 2002; Lee, J. et al., 2002). The timing of DNA demethylation varies according to particular classes of sequences and even within loci of the same sequence class (Walsh et al., 1998; Hajkova et al., 2002; Lee, J. et al., 2002). Although the exact mechanism(s) by which DNA demethylation occurs is currently unknown, recent evidence suggests that the 5-methylcytosine modification is actively removed rather than just passively lost by failure of methylation patterns to be transferred during cell replication (Hajkova et al., 2010; Popp et al., 2010; Cortellino et al., 2011). Active removal of the 5-methylcytosine residue is hypothesized to occur via two steps – deamination of 5-methylcytosine to thymidine by enzymes such as AID followed by TDG-mediated base excision repair of thymidine (Feng et al., 2010; Cortellino et al., 2011).

The loss of DNA methylation is believed to be linked to the incipient expression of post-migratory, gonocyte-specific genes (Maatouk and Resnick, 2003). These genes include *Mvh* (mouse vasa homolog), *GCNA* (germ-cell nuclear antigen), and *Dazl* (deleted in azoospermia-like). The genetic loci of *Mvh* and *Dazl* were found to have high levels of DNA methylation in somatic cells and very low levels of DNA methylation in post-migratory germ

cells. Furthermore, in *Dnmt1* mutant embryos, the loss of DNA methylation leads to precocious expression of GCNA, *Mvh,* and *Dazl* in migratory germ cells (Maatouk et al., 2006).

Dazl is an RNA-binding protein that acts as a competence factor to promote the responsiveness of germ cells to feminizing or masculinizing signals in the environment (Gill et al., 2011). The functions of *Mvh* and *GCNA* in mouse germ-cell development remain unknown.

Role of miRNAs in early germ-cell development

To assess the roles of miRNAs in early germ-cell development, Hayashi and colleagues conditionally deleted *Dicer* in the germline using *TNAP-Cre* (Hayashi et al., 2008). *TNAP-Cre* becomes active around E10, when PGCs are still migrating to the gonad (Lomeli et al., 2000). Conditional deletion of Dicer after E10 resulted in a loss of germ cells due to decreased PGC proliferation. Hayashi and colleagues estimated that 69% and 14% of miRNAs in PGCs were derived from *mir-17-92* and *mir-290-295* respectively, suggesting that loss of these miRNAs might be responsible for the phenotype.

Sexual determination

Although male and female gonads can be morphologically distinguished by E12.5, the germ cells themselves do not display morphological differences until E13.5 when chromosome condensation occurs in female, but not male, germ cells (McLaren, 1983). This difference in nuclear morphology arises from the fact that female germ cells will soon undergo meiosis while male germ cells will instead undergo mitotic arrest. These observations, along with others, led to a model where the decision to become either oocyte or sperm is determined by when the germ cells enter meiosis (Bowles and Koopman, 2010; Gill et al., 2011). If a germ

cell enters meiosis around E13.5, prior to birth, it is committed to undergo oogenesis. In contrast, if a germ cell delays meiosis until after birth, it is obligated to undergo spermatogenesis.

Interestingly, the binary decision to become either oocyte or sperm is not made by the sex chromosome constitution of the germ cells (XY or XX) but rather the sexual identity of the gonadal environment that the germ cells enter (Cattanach, 1987; Taketo-Hosotani et al., 1989; Palmer and Burgoyne, 1991; Adams and McLaren, 2002). The future gonads form around E10 and are considered bipotential until *Sry* (Sex-determining region Y) is expressed in a group of somatic cells around E10.5-E12.5 in the XY gonad (DeFalco and Capel, 2009). These *Sry*-expressing cells will become the Sertoli cells of the testis and will provide support to the germ cells.

The clues to understanding meiotic initiation and thereby sexual determination came from studies on a gene called *Stra8* (stimulated-by-retinoic-acid gene 8). *Stra8* was found to be required for meiotic initiation in both sexes (Baltus et al., 2006; Anderson et al., 2008) and retinoic acid signaling was found to be required for *Stra8* expression (Bowles et al., 2006; Koubova et al., 2006). However, retinoic acid is present during fetal stages in both male and female embryos. Therefore, if retinoic acid is able to trigger meiosis why do male germ cells not undergo meiosis during fetal stages? Furthermore, since retinoic acid is also present earlier in development, why do both female and male germ cells not initiate meiosis prior to their arrival in the gonad? Therefore additional extrinsic/and or intrinsic factors must also regulate meiosis initiation.

Further experiments identified the RNA-binding protein DAZL(deleted in azoospermia-like) as the intrinsic licensing factor required for germ cells to respond to feminizing or masculinziing cues in their gonadal environment (Lin et al., 2008; Gill et al., 2011). *Dazl* expression begins in germ cells around the time of their arrival in the gonad, E10.5-E11.5.

Dazl-deficient germ cells (on a pure C57BL/6 background) do not exhibit any indications of meiotic initiation or gametogenesis.

Since *Dazl* is expressed in both male and female germ cells, there must be other factors that prevent meiotic initiation in males. One such factor is *Cyp26b1*, a member of the cytochrome P450 class of enzymes, which degrades retinoic acid. *Cyp26b1* is greatly upregulated in the testis and downregulated in the ovary (Menke and Page, 2002; Bowles et al., 2006; Koubova et al., 2006). Therefore, although male embryonic germ cells express *Dazl*, *Cyp26b1* expressed in the surrounding somatic cells prevents the germ cells from experiencing retinoic acid signaling. Thus, *Stra8* is not prematurely expressed and the male germ cells do not precociously enter meiosis.

The mechanisms by which meiosis resumes in the adult male after puberty are not well understood although evidence exists that *Dmrt1,* expressed in the germ cells, helps to regulate their responsiveness to retinoic acid (Matson et al., 2010). In addition, it is also unclear how the initial expression of *Sry* in the embryonic XY gonad ultimately relates to inhibition of premature meiosis in the male.

Oogenesis

Around E13.5, as a result of the sex determination process described above, female germ cells initiate meiosis (McLaren, 1983). Meiosis does not progress synchronously but rather in a wave from anterior to posterior (Bowles and Koopman, 2010). The gonocytes, now referred to as oogonia, proceed through prophase I and arrest at the diplotene stage with the first oocytes arresting around E17.5 and the last oocytes arresting by postnatal day 5 (P5) (Borum, 1961). Immediately after birth, the germ-cell cysts begin to break down (Pepling, 2006). Not all oocytes survive cyst breakdown and most die by apoptosis. Oocytes that survive are each surrounded by a layer of squamous pre-granulosa cells and are called

primordial follicles (Pepling, 2006). Because the primordial follicles are arrested in meiosis and cannot self-renew, the primordial follicle pool represents the total number of germ cells the female will have. This primordial follicle pool is a resting population from which oocytes are recruited for growth and maturation.

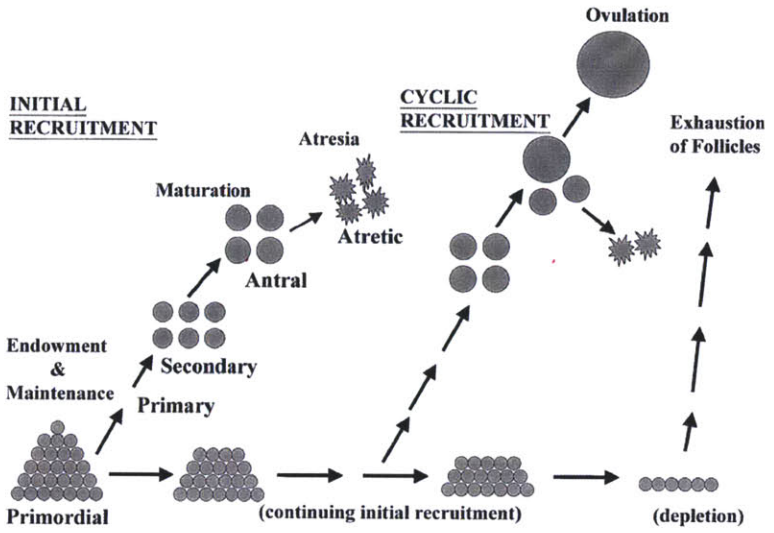

Figure 1.6. Life history of ovarian follicles. Taken from McGee and Hseuh 2000.

During the process of initial recruitment some primordial follicles are selected, by mechanisms unknown, to leave the primordial pool and initiate growth (McGee and Hsueh, 2000; Edson et al., 2009). Those follicles not selected remain dormant. This initial recruitment phase occurs continuously starting right after follicle formation and well before the onset of puberty. Upon leaving the primordial pool, the follicle will enlarge due to an increase in the size of the oocyte and the conversion of the surrounding granulosa cells from a squamous to a cubiodal shape. The transition from a primary follicle to a preantral (secondary) follicle is characterized by an increase in the number of granulosa layers surrounding the oocyte and the addition of a theca cell layer outside the granulose cells. At this stage cross-talk between the oocyte and the surrounding somatic layers is critical for

further maturation of the follicle (Eppig et al., 2002). The next transition to the antral stage of folliculogenesis is characterized by the appearance of small fluid-filled spaces in the follicle that coalesce to form one large antral cavity.

The fate of the antral follicle is decided by the presence or absence of follicle-stimulating hormone (FSH) (McGee and Hsueh, 2000). If follicle-stimulating hormone is not present the antral follicles will degenerate by a process known as atresia. However, after puberty, the increased circulating levels of FSH that occur during each reproductive cycle will rescue a group of antral follicles from atresia, thereby allowing them to be cyclically recruited to the preovulatory stage. As initiation of ovulation proceeds, one follicle gains dominance over the others. The circulating levels of FSH decrease while the levels of luteinizing hormone (LH) surge (Edson et al., 2009). Due to the LH surge, the oocyte resumes meiosis, and the follicle ruptures depositing the oocyte into the oviduct.

Spermatogenesis

Male germ cells enter mitotic arrest between E12.5 and E14.5 depending on the strain background of the mice (Western et al., 2008) and resume mitosis shortly after birth (de Rooij, 1998). Around postnatal day 3 to 6 these immediate precursors of spermatogonia migrate away from the center of the seminiferous tubules toward the periphery and settle on the basement membrane (Bellve et al., 1977). These immediate precursors of spermatogonia are composed of two populations – germ cells destined to become the self-renewing spermatogonial stem cells and germ cells that will differentiate immediately, contributing to the first wave of spermatogenesis (Yoshida et al., 2006). The existence of these two populations was confirmed by experiments tracing the descendants of *Ngn3* (neurogenin 3)-expressing cells. These lineage-tracing experiments revealed that all sperm,

except for those derived in the first wave of spermatogenesis, pass through an *Ngn3*-positive stage.

Those germ cells that do not contribute to the first wave of spermatogenesis will remain at the basement membrane of the seminiferous tubules and become the self-renewing stem cell population that will give rise to differentiating cells and thereby support continued spermatogenesis throughout the life of the animal. Spermatogenesis is a very ordered process with all types of spermatogonia located at the basement membrane of the seminiferous tubules and their descendants arranged in a sequential order towards the lumen. As the spermatogonia divide, they can remain connected by intracellular bridges and from chains (de Rooij, 1998). These undifferentiated spermatogonia can be grouped into several categories based on chain length, A_{single} (A_s), A_{paired} (A_{pr}) and $A_{aligned}$ (A_{al}, 4 to 32 cells). All these three types of cells have the ability to repopulate the empty seminiferous tubules of an infertile male mouse and therefore can be considered stem cells (de Rooij and Russell, 2000).

Spermatogonial stem cells begin differentiation about every 9 days in the mouse (Oakberg, 1956). The first step in differentiation starts when A_{al} cells transition to A_1 cells. This transition is a major regulatory step in spermatogenesis and many factors such as vitamin A (van Pelt et al., 1995) and temperature (Nishimune and Haneji, 1981; de Rooij et al., 1999) can affect this process. The A_{al} to A_1 conversion is marked by morphological changes in the cell but does not involve a mitosis step (Chiarini-Garcia and Russell, 2001). The A_1 spermatogonia then undergo 5 more mitoses to produce A_2, A_3 and A_4 cells. The A_4 cells differentiate into Intermediate and then B type spermatogonia. Although it is hard to distinguish among the A_1-A_4 type of spermatogonia, type A, intermediate, and B spermatogonia can be distinguished from one another based on their chromatin morphology. (de Rooij and Russell, 2000)

Type B cells progress to the pre-leptotene stage where they undergo premeiotic replication and then enter leptotene. The progression from meiotic metaphase through the second meiotic division occurs rapidly in mice (Oakberg, 1956). The last meiosis results in 4 haploid germ cells known as spermatids. These spermatids will then undergo a process called spermiation which takes about two weeks to complete and involves formation of the acrosome, histone-protamine exchange, and flagellum production. Spermatozoa are then released from the testes and travel through the efferent duct to the epididymis. Spermatozoa lack motility until they reach the epididymis and only become active when they enter the female reproductive tract (Abou-haila and Tulsiani, 2009).

PART IV: Functions of the *mir-290-295* Cluster in Embryonic Stem Cells

Introduction to *mir-290-295* cluster

The *mir-290-295* cluster was first discovered in a screen for miRNAs involved in early developmental transitions (Houbaviy et al., 2003). Because *in vitro* differentiation of mouse embryonic stem cells can recapitulate many of the molecular events occurring during early preimplantation development (Arnold and Robertson, 2009), mouse embryonic stem (mES) cells were used as a substitute for the early embryo. This screen revealed a group of miRNAs derived from six pre-miRNAs (*mir-290* - *mir-295*) which mapped to the same 2.2 kb genomic region (Houbaviy et al., 2003). These miRNAs were highly expressed in mouse ES cells but their expression decreased upon mouse ES cell differentiation. Furthermore, northern analysis showed that expression of these miRNAs was undetectable in adult

organs. Therefore, the *mir-290-295* cluster was termed the "Early Embryonic microRNA Cluster" (or EEmiRC).

The *mir-290-295* genomic cluster is located on mouse chromosome 7 and actually encodes seven pre-miRNAs: *mir-290*, *mir-291a*, *mir-292*, *mir-291b*, *mir-293*, *mir-294* and *mir-295* (Fig. 1.7). (Additional analysis indicated another pre-miRNA, *mir-291b*, located between *mir-292* and *mir-293* (Houbaviy et al., 2005).) The genomic sequence of the *mir-290* cluster can be subdivided into two hemiclusters (*mir-290* - *mir-291b* and *mir-293* - *mir-295*) separated by a putative enhancer element (Tata et al., 2011). However, northern analysis indicates that all of these pre-miRNAs are encoded by one long RNA polymerase II primary transcript. An intron located between the transcription start site and *mir-290* is preferentially spliced prior to Drosha processing (Houbaviy et al., 2005).

B

miRNA	mature miRNA sequence
miR-290-5p	acucaaacuaugggggcacuuu
miR-290-3p	aaagugccgccuaguuuuaagccc
miR-291a-5p	caucaaaguggaggcccucucu
miR-291a-3p	aaagugcuuccacuuugugugc
miR-292-5p	acucaaacuggggcucuuuug
miR-292-3p	aaagugccgccaggguuuugagugu
miR-291b-5p	gaucaaaguggaggcccucucc
miR-291b-3p	aaagugccgccaggguuuugagugu
miR-293	agugccgcagaguuuguagugu
miR-293*	acucaaacugugugacauuuug
miR-294	aaagugcuucccuuuugugugu
miR-294*	acucaaaauggaggcccuaucu
miR-295	aaagugcuacuacuuuugagucu
miR-295*	acucaaaauguggggcacacuuc

Figure 1.7. *mir-290-295* cluster. A. Genomic organization of cluster. B. Sequences of mature miR-290 family miRNAs. Seed sequence is in bold.

Orthologs of the entire *mir-290* cluster are present only in eutherian (placental) mammals. For this analysis, in order to be considered an ortholog, the locus needed to meet two criteria (Houbaviy et al., 2005). First, the locus was required to contain one or more pre-miRNA hairpins that matched the multiple sequence alignments of the experimentally indentified murine pre-miRNA hairpins. Second, the locus needed to contain the minimal TATA box

promoter identified in the murine *mir-290* locus. Based on these criteria, in humans, the cluster encoding miR-371, miR-372, and miR-373 is orthologous to the mouse *mir-290* cluster (Suh et al., 2004; Houbaviy et al., 2005). Even though *mir-290* orthologs exist, there is a great deal of sequence variation among orthologs. For instance, the sequences of the orthologous murine *mir-290* and human *mir-371* clusters vary enough to warrant different numerical designations (Ambros et al., 2003). The only conserved elements among *mir-290* orthologs are the minimal promoter and pre-miRNA hairpins (Houbaviy et al., 2003; Houbaviy et al., 2005). Even the actual number and precise sequence of the pre-miRNAs varies among species.

Since the mir-290 cluster encodes seven pre-miRNA hairpins, the entire cluster can potentially generate 14 mature miRNAs (Fig. 1.7). Evidence suggests that these miRNAs are not a homogenous group with respect to expression level and biological function. Work on the miR-290 miRNAs in mouse ES cells has shown that some miRNAs such as miR-291a-3p, miR-292-3p, miR-294 and miR-295 are more highly expressed than other members of the miR-290 family (Babiarz et al., 2008; Ciaudo et al., 2009). Because the *mir-290* cluster is transcribed as a single transcript, posttranscriptional events must explain the differential expression. Although the AAGUGC seed sequence is the most common among miR-290 family members not all of the miR-290 family miRNAs have the same seed sequence, suggesting that different members of this cluster might have different regulatory targets.

The *mir-290* cluster is expressed not only in embryonic stem cells, but also in the early embryo, and primordial germ cells. However, almost all of the current information regarding *mir-290-295* function comes from studies done in embryonic stem cells. Knowledge of the role of embryonic stem cell transcriptional machinery is necessary for understanding the current literature on the function of miR-290 family members.

MiRNA functions in ES Cells

Embryonic stem (ES) cells are derived from the inner cell mass of blastocyst-stage embryos (Evans and Kaufman, 1981; Martin, 1981). ES cells can self-renew and are pluripotent which means that they have the ability to generate all cell types of the embryo proper. Three transcription factors, OCT4, SOX2, and NANOG, work coordinately to maintain the ES cell program. These transcription factors are also involved in the establishment of the pluripotent state. *Oct4* and *Sox2*, along with *Klf4* and *c-Myc* can reprogram murine fibroblasts to an induced pluripotent stem (iPS) cell state (Takahashi and Yamanaka, 2006; Wernig et al., 2007) and *Oct4*, *Sox2*, *Nanog*, and *Lin28* were the four factors first shown to reprogram human fibroblasts to an iPS cell state (Yu et al., 2007).

In both mouse and human ES cells, OCT4, SOX2, and NANOG bind to the promoters of both active and inactive genes (Boyer et al., 2005; Loh et al., 2006). The active genes include genes that are preferentially, but not necessarily exclusively, expressed in ES cells and the early embryo. This trio of transcription factors binds to the promoters of its own genes thus setting up a feed-forward auto-regulatory loop. The inactive genes, whose promoters are also bound by Polycomb group proteins and have a characteristic bivalent domain chromatin structure, mostly include lineage-specification genes (Boyer et al., 2005; Bernstein et al., 2006; Boyer et al., 2006). These genes are activated and transcribed upon differentiation. A more recent study of the binding sites of 13 transcription factors in mouse ES cells revealed additional layers of complexity to the networks maintaining the pluripotent state, particularly how these transcriptional networks interface with extracellular signals (Chen et al., 2008). OCT4, SOX2, and NANOG along with SMAD1 and STAT3 bind a similar group of promoters while N-MYC, C-MYC, E2F1, and ZFX bind to another set of promoters. Exactly how these groups of transcription factors regulate expression of their bound genes

and how these genes contribute to overall ES cell maintenance still remains to be determined.

The trio of transcription factors, OCT4, SOX2, and NANOG along with TCF3 are bound to the promoters of 20% of all annotated mammalian microRNA loci (Marson et al., 2008). Once again these promoters can be classified into two types: promoters of miRNA loci expressed in ES cells and promoters of miRNA loci repressed in ES cells but expressed in specific, differentiated cell types. The promoters of repressed miRNA loci are also bound by Polycomb group proteins, suggesting that these miRNAs are silenced in ES cells but poised to be expressed later on in development. These data suggest that miRNAs play key roles in maintaining the pluripotent state as well as promoting differentiation.

Genetic disruption of key members of the microRNA biogenesis pathway supports this hypothesis. *Dicer* deficiency causes embryonic lethality in the mouse at E7.5 (Bernstein et al., 2003). Furthermore, *Dicer-/-* ES cells could not be derived from *Dicer-/-* blastocysts. Instead, two independent groups used conditional gene targeting to derive *Dicer-/-* ES cells (Kanellopoulou et al., 2005; Murchison et al., 2005). Although *Dicer* was not required for *in vitro* ES cell viability, *Dicer-/-* ES cells failed to differentiate. They could not contribute to chimeras, form teratomas, or undergo embryoid body (EB)-mediated differentiation in contrast to their wild-type counterparts. *Dicer-/-* ES cells grown for 5 days without LIF (leukemia inhibitory factor), a growth factor required for ES cell self-renewal, still showed elevated levels of *Oct4* in contrast to control ES cells grown without LIF. This suggests that perhaps *Dicer* supports differentiation by indirect silencing of *Oct4*. In fact, *Dicer-/-* ES cells showed loss of heterochromatic chromatin modifications as well as lower levels of DNA methylation at minor satellite repeats. However, these observations were reported by only one of the two groups that generated the *Dicer-/-* ES cells (Kanellopoulou et al., 2005).

Because *Dicer* is also required for manufacture of other small RNAs, it was unclear whether the effects of *Dicer* deficiency in ES cells were truly due to loss of miRNAs. The generation of *Dgcr8-/-* ES cells helped to resolve these possibilities since DGCR8 is only required for (canonical) miRNA processing. *Dgcr8-/-* cells maintained ES cell characteristics, such as *Oct4* and *Nanog* expression, but showed defects in cell proliferation and EB differentiation (Wang et al., 2007). However, these defects were more subtle than those observed in the *Dicer-/-* ES cells. *Dgcr8-/-* ES cells showed a less profound defect in the G1-S transition compared to *Dicer-/-* ES cells. In addition, while *Dicer-/-* ES cells showed no expression of differentiation markers during EB differentiation, *Dgcr8-/-* ES cells displayed abnormal but not complete loss of differentiation markers. Interestingly, in the *Dgcr8-/-* cells, silencing of pluripotency genes, *Oct4*, *Sox2*, and *Nanog* was defective during retinoic acid differentiation. These observations suggest that miRNAs might somehow help to silence pluripotency genes and thereby support differentiation of ES cells.

What are the miRNAs that might be mediating these effects? *Mir-290-295* and five other genomic loci, *mir-17-92*, *mir-21*, *mir-15b/16*, a repetitive cluster on chromosome 2 and an imprinted cluster on chromosome 12, account for the majority (73%) of miRNAs in mouse ES cells (Calabrese et al., 2007). The *mir-290* cluster is the most abundant in mouse ES cells, representing about 25% of total miRNAs and is followed closely by *mir-17-92*, representing about 14% of total miRNAs in ES cells (Calabrese et al., 2007; Babiarz et al., 2008; Marson et al., 2008; Ciaudo et al., 2009).

Surprisingly, the *mir-290* human ortholog, the *mir-371* cluster, is not the most highly expressed cluster in human ES cells. Instead, the *mir-302* cluster on chromosome 4 (*mir-302b*, *mir-302c*, *hsc-3(mir-302)*, and *mir-302d*) is the most highly expressed (Suh et al., 2004). The mouse ortholog to these human miR-302 miRNAs, mmu-miR-302, is less abundant in mouse ES cells (Houbaviy et al., 2003; Calabrese et al., 2007; Babiarz et al.,

2008; Marson et al., 2008; Ciaudo et al., 2009). Interestingly, both the miR-302 family miRNAs and many members of the miR-290 family share the AAGUGC seed sequence.

Studying the functions of embryonic stem cell microRNAs, particularly miR-290 family members, uncovered their roles in cell-cycle progression, apoptosis, DNA methylation, and reprogramming.

Role of miR-290 family members in ES cell-cycle progression

The cell proliferation defects observed in *Dicer* and *Dgcr8*-deficient ES cells hinted that ES cell miRNAs play a role in the unique cell-cycle structure of pluripotent cells. The canonical cell cycle is divided into 4 consecutive phases: DNA replication followed by a gap (G2) phase, followed by mitosis, and followed by another gap (G1) phase prior to the start of another round of DNA replication. In contrast to differentiated cells, embryonic stem cells have an extremely short G1 phase and spend most of their time (60%) in S phase (Mac Auley et al., 1993; Stead et al., 2002). Because of these shortened gap phases, mouse ES cells have a rapid cell-cycle time of approximately 12 hrs, compared to 24 hours for differentiated cells (Savatier et al., 1994).

What is the mechanism behind the short G1 and rapid cell-cycle times of mouse ES cells? In somatic cells, transition from G1 to S requires passing through the restriction (R) checkpoint, after which the cell cycle becomes growth factor independent (Orford and Scadden, 2008). Progress through the G1 phase of the cell cycle is controlled by the action of cyclin-Cdks and their effects on the Retinoblastoma protein, Rb (Harbour et al., 1999). *Hypo*phosphorylated Rb is able to block S-phase entry by binding to and sequestering E2F transcription factors which promote the transcription of S-phase genes. Cyclin-Cdks promote

progression from G1 to S by phosphorylating Rb, which causes the release of E2F proteins (Orford and Scadden, 2008).

In differentiated cells the expression of cyclins oscillates throughout the cell cycle. However, in pluripotent cells, cdk2-cyclin E/A and cdk6-cylin D3 are present in high amounts throughout the cell cycle (Stead et al., 2002). These Cdks keep Rb in a constant hyperphosphorylated state (Savatier et al., 1994). Furthermore, ES cells also show an absence of cell-cycle inhibitors such as p16ink4a and p27kip. All these factors contribute to lack of a restriction point in ES cells and the rapid cell cycle (White and Dalton, 2005).

Because of the G1 accumulation observed in *Dgcr8-/-* (and *Dicer-/-*) ES cells, Wang and colleagues hypothesized that embryonic stem cell miRNAs repress inhibitors of the G1-S transition (Wang et al., 2008). To test their hypothesis, Wang and colleagues screened 266 miRNAs for their ability to rescue the proliferation defect of *Dgcr8-/-* ES cells. Thirteen miRNAs including miR-291a-3p, miR-291b-3p, miR-294 and miR-295 and miR-302b, c, and d, were found to rescue the proliferation defect to various degrees. Since the miR-290 family members are the highest expressed miRNAs in mouse ES cells, and miR-302 in human ES cells, the researchers chose to focus their efforts on miR-291a-3p, miR-291b-3p, miR-294, miR-295 and miR-302. These five miRNAs share a similar seed sequence (AAGUGC) and were each able to restore the characteristic wild-type cell-cycle profile to *Dgcr8-/-* ES cells. The researchers identified *p21*, an inhibitor of cyclin E-Cdk2, as a direct, functional target of these five miRNAs. Other inhibitors of cyclin E-Cdk2 complex, *Rbl1, Rbl2*, *Lats2*, were also indicated as targets. Therefore, miR-290 family members miR-291a-3p, miR-291b-3p, miR-294, and miR-295 promote the characteristic rapid proliferation of ES cells by repressing inhibitors of cyclin E-Cdk2, an inducer of the G1-S transition.

Role of *mir-290-295* in apoptosis

miR-290 miRNAs also protect ES cells from apoptosis during exposure to genotoxic stress (Zheng et al., 2011). Compared to their wild-type counterparts, *Dicer-/-* and *mir-290-295-/-* ES cells exhibit increased apoptosis after exposure to either irradiation or the DNA-damaging agent doxorubicin. Zheng and colleagues began to uncover the mechanism behind this phenomenon by comparing the list of genes upregulated in *Dicer-/-* and *mir-290-295-/-* ES cells (relative to wild-type) with computationally predicted targets for miR-290 AAGUGC seed miRNAs . *Caspase 2* was a top predicted target; its 3'UTR contained multiple sites for AAGUGC seed binding. Another apoptotic gene, *Ei24*, was also found to be a direct target of miR-290 AAGUGC seed miRNAs. Addition of miR-290-3p or miR-295 to *mir-290-295-/-* ES cells decreased *Caspase 2* and *Ei24* levels and resulted in reduced apoptosis after ES cell exposure to genotoxic stress. This study provides a clear example of how miR-290 miRNAs, by modulating a stress response, might contribute to robustness.

Role of *mir-290-295* in DNA methylation

The miR-290 family was also implicated in ES cell differentiation through its role in DNA methylation. *Dicer*-deficient ES cells show reduced levels of the *de novo* DNA methyltransferases *Dnmt3a*, *Dnmt3b*, *Dnmt3L* (Benetti et al., 2008; Sinkkonen et al., 2008). Introduction of miR-290 family miRNAs into *Dicer-/-* ES cells restored the de novo DNA methyltransferases to wild-type levels. The RBL2 (retinoblastoma like 2) protein, which negatively regulates *Dnmt3a*, *Dnmt3b*, and *Dnmt3L*, is a direct target of miR-290 family miRNAs. Presumably, loss of *mir-290-295* results in an increase in *Rbl2* which then represses transcription of *Dnmt3a*, *Dnmt3b*, and *Dnmt3L* genes. This pathway could explain the differentiation defects in *Dicer* and *Dgcr8* deficient ES cells. *Dicer-/-* ES cells fail to stably silence *Oct4* during retinoic acid-induced differentiation (Benetti et al., 2008; Sinkkonen et al., 2008). Although repressive histone marks are present at the *Oct4* promoter in differentiating

ES cells, DNA methylation is absent. Introduction of miR-290 family members into *Dicer* deficient ES cells that are then differentiated results in wild-type DNA methylation levels at the *Oct4* promoter. If this defect extends to many of the pluripotency-specific genes it could explain how global loss of miRNAs prevents proper ES cell differentiation. However, miR-290 miRNAs do not actually rescue the differentiation effects of *Dgcr8-/-* ES cells (Wang et al., 2008; Melton et al., 2010).

Role of *mir-290-295* in promoting the ES cell state

Further evidence suggests that, rather than promote differentiation, members of the miR-290 family promote self-renewal by reinforcing mechanisms to prevent let-7 expression (Melton et al., 2010). Let-7 is highly expressed in differentiating cells. Therefore, intrigued by the inability of *Dgcr8-/-* ES cells to undergo successful differentiation, Melton and colleagues hypothesized that let-7 might silence the self-renewal of miRNA-deficient ES cells. When *Dgcr8-/-* cells cultured under ES cell conditions were transfected with let-7 mimic, these cells demonstrated decreased *Oct4*, *Sox2*, and *Nanog* expression as well as a reduced capacity to re-form ES colonies after passaging.

Surprisingly, addition of let-7 mimic to wild-type ES cells was not sufficient to silence self-renewal (Melton et al., 2010). The researchers then proposed that miRNAs in ES cells, particularly the highly expressed miR-290 family, might be counteracting the effects of exogenous let-7 in the wild-type ES cells. Several members of the miR-290 family, miR-291a-3p, 291b-3p, 294, and 295 were found to block the let-7 -mediated silencing of self-renewal in *Dgcr8-/-* ES cells. (Interestingly, the other members of the mR-290 family, miR-292 and miR-293 were not able to counteract the effect of let-7).

The proposed model based on these data suggests that miR-290 family members and let-7 act in opposing regulatory networks to enforce either the pluripotent state (miR-290

members) or the differentiated state (let-7). MiR-290 family members (291a-3p, 291b-3p, 294 and 295) indirectly upregulate *Lin28* and *c-Myc* (Melton et al., 2010). Since LIN28 blocks maturation of *let-7* (Heo et al., 2008; Newman et al., 2008; Rybak et al., 2008; Viswanathan et al., 2008) the miR-290 members ultimately prevent expression of mature let-7. As ES cells differentiate, the expression of *Oct4* and *Nanog* decreases, which subsequently decreases transcription of the *mir-290* cluster. In turn, *Lin28* is downregulated and mature let-7 becomes expressed. *Lin28* is further decreased by *let-7* and *let-7* also inhibits the downstream genes positively regulated by *Oct4* and *Nanog,* thereby helping to promote the differentiated state.

Role of *mir-290-295* in somatic cell reprogramming

In addition to supporting the pluripotent state, miR-290 family members can also promote induction of a pluripotent stem cell state. miR-294 (and to some extent miR-291-3p and miR-295) were able to substitute for, but not enhance, *c-Myc's* contribution to reprogramming efficiency (Judson et al., 2009). However the effects of *c-Myc* and miR-294 on reprogramming were not identical. Unlike *c-Myc*, miR-294 did not promote proliferation of MEFs early in reprogramming; however, with miR-294, a more uniform (with respect to *Oct4*-positive) population of cells was observed early in reprogramming. Additional studies on miR-294 targets in ES cells, have indicated that miR-294, rather than simply being downstream of *c-Myc*, may promote pluripotency through a shared subset of *c-Myc* target genes (Hanina et al., 2010). Studies in human fibroblasts have shown that miR-372 (an ortholog of miR-290 family members) and miR-302 can also promote four factor (*Oct4*, *Sox2*, *c-Myc*, and *Klf4*)-mediated reprogramming (Subramanyam et al., 2011). Impressively, recent work has also demonstrated that repeated transfection of only a small group of miRNAs, including murine miR-302 family members, can reprogram mouse and human somatic cells

to a pluripotent state, thereby eliminating the need for ectopic protein expression (Anokye-Danso et al., 2011; Miyoshi et al., 2011).

Conclusions

This introduction has covered modes of miRNA function, germ-cell development, and early embryo development. This chapter has concluded with an overview of several studies showing that *mir-290-295* has roles in ES cell-cycle progression, prevention of apoptosis, DNA methylation and maintenance of the ES cell state. However, the *in vivo* function of *mir-290-295* is still unknown. In this thesis, we explore the consequences of *in vivo mir-290-295* deficiency in the mouse.

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Chapter 2

Mir-290-295 Deficiency in Mice Results in Partially Penetrant Embryonic Lethality and Germ-Cell Defects

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L.M.D. generated *mir-290-295-/-* mice.
L.M.D., S.M., and O.K derived *mir-290-295-/-* embryonic stem cells.

Personal Contribution: Determined time range of embryonic lethality. Discovered embryonic developmental delay and out-of-yolk-sac phenotype. Analysis of germ cell phenotype along with Mark Gill. Wrote Manuscript and prepared all Figures and Tables with the exception of Figures 1 and 2.

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Summary

mir-290 through *mir-295* (*mir-290-295*) is a mammalian-specific miRNA cluster that, in mice, is expressed specifically in early embryos and embryonic germ cells. Here, we show that *mir-290-295* plays important roles in embryonic development as indicated by the partially penetrant lethality of mutant embryos. In addition, we show that in surviving *mir-290-295*-deficient embryos, female but not male fertility is compromised. This impairment in fertility arises from a defect in migrating primordial germ cells and occurs equally in male and female mutant animals. Male *mir-290-295-/-* mice, due to the extended proliferative lifespan of their germ cells, are able to recover from this initial germ-cell loss and are fertile. Female *mir-290-295-/-* mice are unable to recover and are sterile, due to premature ovarian failure.

Introduction

In the mouse, miRNA-mediated posttranscriptional regulation is required for normal embryogenesis (Bernstein et al., 2003; Liu et al., 2004; Wang et al., 2007) and embryonic germ cell development (Hayashi et al., 2008). While critical roles for miRNA biogenesis in early embryos and embryonic germ cells have been established, the role of individual miRNAs in the development of these cell types remains unclear. Six miRNA families comprise the majority of miRNA species cloned from mouse embryonic stem (ES) cells, with miRNAs from the *mir-290* cluster, *mir-290* through *mir-295* (*mir-290-295*), being the most abundant (Calabrese et al., 2007). Members of this cluster are the first embryonic miRNAs up-regulated in the zygote (Tang et al., 2007). It has previously been shown that the *mir-290*

cluster miRNAs are processed from a single primary transcript (Houbaviy et al., 2005) and possess highly similar pre-miRNA sequences (Houbaviy et al., 2003).

Several studies have addressed the role of *mir-290-295* in embryonic stem (ES) cells where this cluster is a direct target of the *Oct4*, *Sox2*, and *Nanog* regulatory network (Marson et al., 2008). The *mir-290* cluster is correlated with developmental potency. *mir-290-295* expression decreases as ES cells differentiate (Houbaviy et al., 2003). Furthermore, certain members of the *mir-290* cluster were found to increase the efficiency of reprogramming by *Oct4*, *Sox2*, and *Klf4* approximately 10 fold (Judson et al., 2009). In addition, members of the *mir-290* cluster promote the G1-S transition and thereby the rapid proliferation characteristic of ES cells (Wang et al., 2008). The *mir-290* cluster was also implicated in indirect control of *de novo* DNA methylation in ES cells (Benetti et al., 2008; Sinkkonen et al., 2008). Taken together, these data imply important roles for *mir-290-295* in ES cells and by extension, early mouse development. In this study, we examined the *in vivo* consequences of targeted disruption of *mir-290-295* in the developing mouse.

Results

mir-290-295 is specifically expressed in the early embryo and embryonic germ cells

We have shown that *mir-290-295* is expressed in ES cells and not in adult somatic tissues (Houbaviy et al., 2003). To address the timing of *mir-290* cluster expression, we performed RT-PCR for the *mir-290-295* primary transcript (*pri-mir-290-295*) throughout early embryonic development on pools of embryos. We observed onset of expression of the primary transcript at the 4 to 8-cell stage (Fig. 2.1A), consistent with the finding that expression of

these miRNAs is upregulated post-zygotically (Tang et al., 2007). Expression of the *mir-290-295* primary transcript decreased after E6.5.

In order to more closely examine the expression of the *mir-290* cluster miRNAs after gastrulation, we performed RT-PCR for the primary transcript on a panel of embryonic tissues from E14.5 embryos. Expression of the *mir-290* cluster was detectable in the embryonic testis but not in any other tissue examined (Fig. 2.1B). Because *mir-290-295* has been shown to be expressed in primordial germ cells (Hayashi et al. 2008), we also analyzed *mir-290* cluster expression in germ cells during embryonic development. Expression of the *mir-290* cluster was observed in gonads of both sexes at E12.5. The *pri-mir-290-295* transcript was downregulated in female gonads between E13.5 and E14.5, while in male gonads expression persisted through E14.5 and became undetectable by E15.5 (Fig. 2.1C).

Since the embryonic gonad is comprised of both somatic and germ cell populations, we examined *mir-290-295* expression in E14.5 W^v/W^v gonads to test whether *mir-290-295* expression in the E14.5 testis was dependent upon the presence of germ cells. W^v homozygotes harbor a mutation in the *c-kit* gene that impairs primordial germ cell (PGC) migration to the gonad, resulting in loss of germ cells prior to E14.5 (Nocka et al., 1990). *Pri-mir-290-295* was undetectable in embryonic W^v/W^v testes, suggesting that *mir-290-295* expression in embryonic gonads is restricted to the germ cells (Fig. 2.1D).

Generation of *mir-290-295* mutant mice

To determine the function of *mir-290-295 in vivo*, we generated mice deficient for the 2 kb locus containing these miRNAs by targeted disruption in ES cells (Fig. 2.2A). Two independent *mir-290-295+/-* ES cell lines, where correct targeting had been validated by Southern blot and PCR, were injected into B6DF2 host blastocysts to produce chimeras. Transmission of the targeted allele through the male germline was confirmed by Southern

blotting and PCR analysis (Fig. 2.2B). To verify that deletion of the *mir-290-295* locus eliminated expression of the miR-290 family of miRNAs, *mir-290-295* homozygous knockout ES cells lines were produced. Northern blot (Fig. 2.2C) and real-time RT-PCR (data not shown) using probes to detect mature miR-290 family miRNAs failed to detect any of these miRNAs in mutant (*mir-290-295-/-*) ES cells, confirming that the targeted deletion resulted in a null allele. These findings suggest that *mir-290-295* is dispensable for maintaining the pluripotent state in embryonic stem cells.

mir-290-295 deficiency results in partially penetrant embryonic lethality

mir-290-295 +/- mice were fertile and indistinguishable from wild-type littermates. When heterozygous animals were intercrossed, we observed a significantly lower fraction of homozygous *mir-290-295-/-* offspring than the 25% predicted by Mendelian segregation (Table 2.1). Only 7% (32 of 452, p=0.001, chi squared) of 4-week-old postnatal progeny from *mir-290-295* +/- intercrosses were *mir-290-295-/-*, suggesting that about three-quarters of the homozygous mutant animals were lost during development. At E18.5, just prior to birth, the percentage of mutant embryos observed (7%, 3 out of 46, p=0.01, chi squared) was identical to that seen at postnatal stages indicating that perinatal lethality was not responsible for the loss of *mir-290-295-/-* embryos (Table 2.1).

To determine when during gestation *mir-290-295-/-* embryos were lost, embryos from heterozygous intercrosses were isolated at blastocyst (E3.5) and mid-late gestation (E8.5-E18.5) stages of development. Mutant (*mir-290-295-/-*) blastocysts appeared morphologically indistinguishable from their wild-type and heterozygous counterparts and were observed at the predicted Mendelian ratio of 25% (32 out of 117 total blastocysts). Further analysis of embryos at mid-late gestation suggested that *mir-290-295-/-* embryos were lost over a period of time between E11.5 and E18.5.

Even though mutant embryos were observed at the predicted Mendelian ratio at E8.5, E9.5, and E10.5, E11.5 and E13.5, approximately 50-60% of these embryos (Table 2.2) displayed abnormalities not observed in their wild type or heterozygous littermates. Two abnormal phenotypes were observed. Prior to E10.5 about 16% of the mutant embryos were partially or completely localized outside the yolk sac (Fig. 2.3). Such abnormal embryos were not observed at later stages presumably because they had died and been reabsorbed.

The second abnormal phenotype, comprising about 40% of the mutant embryos, showed general developmental delays as early as E8.5. These mutants had fewer somites than their wild-type or heterozygous littermates and showed delays in chorioallantoic attachment, axial turning, and neural tube closure.

Adult *mir-290-295 -/-* females are sterile, whereas *mir-290-295-/-* males are fertile

Surviving *mir-290-295-/-* animals were healthy and phenotypically normal, though homozygous mutant females were infertile. Of six *mir-290-295-/-* females caged with fertile wild-type males for up to 12 months, none produced offspring. To explore female infertility further, we examined ovaries from adult (5-12 weeks post partum) *mir-290-295-/-* animals. Adult ovaries were small, having a volume less than 20% that of wild-type littermates (Fig. 2.4A). Ovaries from homozygous mutants older than 10 weeks contained no observable follicular structures (data not shown). In the ovaries of 5-8-week old *mir-290-295-/-* animals, small numbers of follicles were occasionally observed (Fig. 2.4C & E).

mir-290-295-/- males regularly fathered litters with both wild-type and *mir-290-295+/-* females. While testes from adult *mir-290-295-/-* males were relatively similar in size to the testes of wild-type males (Fig. 2.4F), histological periodic acid-Schiff (PAS)-stained sections of *mir-290-295-/-* males showed empty seminiferous tubules (black rectangle in Fig. 2.4H) alongside tubules filled with germ cells at various stages of spermatogenesis (Fig. 2.4H & J).

Both male and female mir-290-295-/- early postnatal gonads show reduced germ cell numbers

Examination of ovaries and testes from postnatal day 5 (P5) animals revealed a reduced number of gonocytes in both males and females. At P5, *mir-290-295-/-* ovaries contained less than 20% as many oocytes as wild-type ovaries as determined by histological analysis and immunostaining for MVH (mouse vasa homolog), a germ-cell marker (Fujiwara et al., 1994) (Fig. 2.S1 A-D). In contrast to the primordial follicles observed in the P5 wild-type ovaries (Fig. 2.S1A & C), most of the follicles observed in the *mir-290-295-/-* ovaries were growing follicles (Fig. 2.S1B & D). Very few primordial follicles (Fig 2.S1D arrows) were observed in the mutant. Consistent with these data, postnatal day 10 (P10) *mir-290-295-/-* ovaries showed severe depletion of the primordial follicle pool (Fig. 2.S1F & H compared to Fig. 2.S1E & G, arrows point to the primordial follicles in the wild-type female). Because the primordial follicle pool is a finite population (Bristol-Gould et al., 2006), once all the follicles have been recruited from the pool for maturation and/or death, the female will no longer be fertile.

mir-290-295-/- testes also showed fewer germ cells than controls at P5 (Fig 2.S1 I-L). Chains of 3-4 gonocytes were observed in P5 homozygous mutant males, suggesting that the male *mir-290-295-/-* germ cells were still proliferating. Furthermore, at P10, non-empty tubules in the homozygous mutant male contained more than the clusters of three to four gonocytes observed at P5, further suggesting that the male *mir-290-295-/-* germ cells continued to proliferate (Fig. 2.S1 M-P). These data are not surprising considering that the expression data in Fig. 2.1C indicated that the *mir-290* cluster is not expressed after E14.5 in males.

Taken together, the above data, combined with the observation of empty seminiferous tubules in the adult male homozygous knockout (Fig. 2.4H & J), suggest that the original

germ cell defect is initially sex-neutral and that males are able to regain fertility by clonal expansion of surviving germ cells. Because of primordial germ-cell depletion, some tubules will have no spermatogonial stem cells and will remain empty. Since the development of male germ cells is characterized by an extended proliferative lifetime relative to that of female germ cells, it seems likely that the male-specific germ-cell recovery observed in *mir-290-295-/-* gonads is a consequence of this sexual dimorphism – rather than a consequence of *mir-290-295* deficiency *per se*.

Both male and female *mir-290-295-/-* embryonic gonads show germ-cell depletion

To further explore the germ-cell deficiency we determined the timepoint during development when a difference in germ-cell number or localization became apparent between homozygous knockouts and their wild-type siblings. Because *mir-290-295* expression becomes undetectable after E13.5 in females and E14.5 in males (Fig. 2.1C), we examined E13.5 gonads by immunostaining for the germ-cell marker MVH. Inspection of *mir-290-295-/-* ovaries and testes at E13.5 revealed a dramatic reduction in the number of MVH-positive cells relative to control embryonic gonads (Fig. 2.5 A-E). To ensure that the loss of MVH expression was indicative of decreased germ-cell number, and not a gene-specific effect of *mir-290-295* deficiency, we examined expression of another germ-cell marker, germ-cell nuclear antigen (GCNA) (Enders and May, 1994) in E13.5 homozygous knockout gonads and found a similar reduction in the number of GCNA-positive cells (data not shown).

We then examined E11.5 embryos to determine whether reduced numbers of *mir-290-295-/-* primordial germ cells (PGCs) were colonizing the genital ridges. At E11.5 most male and female *mir-290-295-/-* genital ridges exhibited less than 5% as many germ cells as found in wild-type littermate controls (Fig. 2.5 F-J), as determined by MVH immunostaining.

mir-290-295-/- animals show many mislocalized primordial germ cells

Based on the reduced number of germ cells colonizing the genital ridges in E11.5 homozygous knockouts and because the *mir-290-295* locus is expressed in migrating primordial germ cells (Hayashi et al., 2008) we explored the possibility that mutant embryos exhibited defective germ-cell migration. Primordial germ cells (PGCs) in the developing mouse embryo migrate from their origin in the proximal epiblast to the genital ridges (Richardson and Lehmann, 2010). Migration begins around E7.5 when PGCs move from the epiblast into the endoderm (Anderson et al., 2000). Around E7.75 PGCs become incorporated into the developing hindgut by possibly active but mostly passive mechanisms (Anderson et al., 2000; Hara et al., 2009). PGCs then actively migrate dorsally through the hindgut into the midline dorsal body wall and subsequently migrate laterally into the mesentery, reaching the genital ridges by E11.5 (Molyneaux et al., 2001; Molyneaux et al., 2003). Not all PGCs successfully migrate to the gonads. Those that remain in the midline undergo apoptosis around E11.5 (Stallock et al., 2003; Runyan et al., 2006).

We employed two different germ-cell markers, tissue non-specific alkaline phosphatase (TNAP or AP) (Ginsburg et al., 1990) and *Oct4*, to locate germ cells in the midgestation embryo. Alkaline phosphatase expression was detected by whole-mount staining while an *Oct4*-GFP transgene was used to monitor *Oct4* expression (Yeom et al., 1996). In wild-type animals at E9.5, PGCs were located almost exclusively in the hindgut and dorsal mesentery (Fig. 2.6A, C & D). Although there was great variability among PGC numbers, both *mir-290-295-/-* males and females had about one-fourth as many germ cells in the hindgut and mesentery as wild-type animals of the same developmental stage (Fig. 2.6B, C & D).

Surprisingly, mutant males and females exhibited AP+ *Oct4*+ cells on the ventral surface of the embryo near the hindlimb buds and the base of the tail (Fig. 2.6B). On average, mutant embryos had 40 of these ectopic PGCs whereas their wild-type counterparts had 10

or fewer (Fig. 2.6D). These ectopic PGCs were observed as late as E11.5 (data not shown). A difference between germ-cell localization in mutants and control littermates could be observed as early as E8.5. In *Oct4*-GFP wild-type embryos at the 7-somite stage, *Oct4*$^{+}$ cells had already moved into the hindgut (Fig. 2.S2A). In contrast, *Oct4*$^{+}$ cells in the 7-somite stage *Oct4*-GFP *mir-290-295-/-* embryos were clustered together at the base of the allantois (Fig. 2.S2B & C). Taken together, these data suggest that loss of germ cells in *mir-290-295-/-* animals is at least partially due to improper germ-cell migration.

mir-290-295-/- germ cells do not undergo premature cell-cycle arrest or apoptosis

Because the *mir-290* cluster has been shown to regulate the G1-S transition (Wang et al., 2008), and protect against apoptosis (Zheng et al., 2011), in ES cells, we explored the possibility that *mir-290-295-/-* germ cells might have undergone apoptosis or premature cell-cycle arrest. We studied two timepoints during germ-cell development: E9.5, when germ cells are migrating toward the developing gonad and E12.5, after germ cells have arrived in the gonad but before they have undergone mitotic arrest (in males) or meiotic arrest (in females). E9.5 embryos were serially sectioned and stained for either Ki-67 or cleaved-caspase-3. In all sections, SSEA-1 was used to identify the migrating PGCs (Fox et al., 1981). At least 90% of both wild-type and *mir-290-295-/-* migrating PGCs were Ki-67 positive (Fig. 2.7 A-G). Ki-67 protein is expressed in all proliferating cells during the late G_1, S, G_2, and M phases of the cell-cycle. Only cells in the G_0 phase of the cell-cycle do not express Ki-67 (Gerdes et al., 1983; Scholzen and Gerdes, 2000). Therefore, the data from E9.5 animals, indicate that *mir-290-295-/-* PGCs are actively cycling. Furthermore, neither wild-type nor *mir-290-295-/-* E9.5 PGCs were cleaved-caspase-3 positive (Fig. 2.7 H-O) indicating that *mir-290-295-/-* PGCs are not undergoing apoptosis.

Similar results were obtained with E12.5 gonocytes. Male and female wild-type and *mir-290-295-/-* E12.5 gonads were serially sectioned and stained for either MVH and PCNA or SSEA-1 and cleaved-caspase-3. PCNA, like Ki-67 is a marker of actively cycling cells (Wrobel et al., 1996). (Because double Ki-67 and MVH immunostaining proved difficult, PCNA was substituted for Ki-67.) At E12.5, in both male and female gonads, at least 85% of the gonocytes were PCNA-positive, and therefore actively cycling (Fig. 2.S3). In addition, neither wild-type nor *mir-290-295-/-* males or females exhibited any cleaved-caspase-3 positive germ cells (Fig. 2.S4). Taken together these data show that decreased numbers of germ cells in the *mir-290-295-/-* animal cannot be explained by apoptosis or failure of the mutant germ cells to proliferate.

Discussion

In this study we investigated the biological function of the *mir-290* cluster by targeted deletion in the mouse. Although miRNAs of the *mir-290* cluster are the first miRNAs upregulated in the developing embryo, this cluster was not required for preimplantation development or ES cell pluripotency. Instead, we found that *mir-290-295* deficiency had a significant effect between implantation and midgestation and during germ-cell development. Approximately three-quarters of *mir-290-295*-deficient embryos were lost during embryonic development. The surviving quarter of homozygous knockouts showed a germ-cell loss. Adult male mutants recover from this loss while female mutants do not and are sterile.

mir-290-295 deficiency confers an incompletely penetrant embryonic lethality

The earliest abnormality in *mir-290-295* homozygous knockouts was observed at E8.5. Specifically, about 16% of *mir-290-295*-deficient embryos at E8.5 (and E9.5, and E10.5) were found either partially or completely outside of the yolk sac. The phenomenon of

postimplantation embryos located either partially or completely outside the yolk sac has been observed for mutants of several genes involved in patterning the embryo during gastrulation. These mutants include the *Nodal* hypomorph (Lowe et al., 2001), *Type II activin receptor* homozygous knockout (Song et al., 1999), *Hnf3β* homozygous knockout (Dufort et al., 1998), *Otx2* homozygous knockout (Ang et al., 1996), *Lpp3* homozygous knockout (Escalante-Alcalde et al., 2003), and *axin* homozygous knockout (Zeng et al., 1997). The exact mechanism by which these embryos end up outside the yolk sac remains unclear and likely varies depending upon the function of the mutated gene. Aberrant cell migration during gastrulation and defective cell proliferation, particularly of the visceral endoderm, are believed to be the two main mechanisms (Dufort et al., 1998; Foley and Stern, 2001).

One intriguing possibility is that the *mir-290-295* cluster might play a role in gastrulation by regulating *lefty1* and *lefty2*. Both *lefty1* and *lefty2* have been shown to be targets of the *mir-290-295* cluster (Marson et al., 2008; Sinkkonen et al., 2008). In zebrafish, miR-430, which has the same AAGUGC seed sequence as many of the miRNAs in the miR-290 family, balances the expression of the Nodal agonist *squint* and the TGF-β nodal antagonist *lefty* (Choi et al., 2007). Recent work suggests human miRNAs, homologous to zebrafish miR-430 and mouse miR-290-295, might function similarly. In human ES cells depletion of *mir-302*, which targets *lefty1* and *lefty2*, leads to a strong decrease in the expression of mesodermal and endodermal markers (Rosa et al., 2009). The *mir-290-295* mutant mouse would provide a unique opportunity to test the hypothesis that AAGUGC seed miRNAs are involved in balancing *lefty-Nodal* signaling.

mir-290-295 deficiency results in germ-cell loss

Approximately 25% of *mir-290-295-/-* animals survived to adulthood. Although adult female homozygous knockouts were sterile and male homozygous knockouts were fertile, the germ-

cell loss leading to sterility in the female was first observed in *both* sexes at E11.5. This suggests that fewer germ cells were colonizing the gonads in both *mir-290-295-/-* females and males. Observations of migrating PGCs revealed a reduction in the number of correctly localized germ cells in *mir-290-295*-deficient animals. In E9.5 male and female mutants, the decreased number of PGCs properly localized to the hindgut area correlated with the increased number of ectopic germ cells observed on the ventral posterior surface of the embryo. These data suggest that the germ-cell loss observed in the mutant is due to mislocalization of a subpopulation of primordial germ cells, which are subsequently unable to colonize the gonad and therefore cannot contribute to the germ-cell pool.

Given that members of the *mir-290-295* family have been shown to regulate the G1-S transition (Wang et al., 2008), and recently apoptosis (Zheng et al., 2011), in ES cells, we investigated whether increased apoptosis or a block in proliferation could explain the germ-cell loss in the mutant animals. No cleaved-caspase-3-positive PGCs were observed in wild-type or *mir-290-295-/-* germ cells at E9.5 or E12.5. In E9.5 animals, at least 90% of migrating PGCs in both wild-type and *mir-290-295-/-* animals were actively cycling. In E12.5 animals, at least 85% of both male and female wild-type and mutant gonocytes were actively cycling. Whereas the above results rule out the possibility of premature cell-cycle arrest in *mir-290-295-/-* PGCs, they cannot exclude the possibility that slower proliferation kinetics of the mutant germ cells contributes to the initial decrease in germ cells caused by mislocalization

Nevertheless, whereas a defective G1-S transition might be able to explain part of the germ-cell loss realized at E11.5 and E13.5, it would not explain the primary observation that some *mir-290-295-/-* germ cells are mislocalized during migration. Ectopic PGCs localized in or near the tail have been previously reported in mice deficient for the pro-apoptotic protein Bax (Stallock et al., 2003; Runyan et al., 2006; Runyan et al., 2008). While the majority of

ectopic PGCs in *Bax-/-* mice were found within the abdominal midline dorsal body wall, some ectopic PGCs were also observed on the tail. Unlike *Bax-/-* embryos, *mir-290-295-/-* embryos do not display ectopic germ cells in or near the abdominal midline body wall, suggesting that mislocalization of *mir-290-295-/-* germ cells is not simply a consequence of faulty apoptotic pathways.

Ectopic "tail" PGCs are hypothesized to arise from PGCs which fail to become incorporated into the hindgut during the initial stages of germ-cell migration (Runyan et al., 2008). Our observations are consistent with this model. At E8.5, while wild-type PGCs have already entered the developing hindgut, the majority of homozygous mutant PGCs have not yet started to migrate and instead are stuck near the base of the allantois (Fig. 2.S2). Whether the failure of germ cells to disperse by E8.5 is due to cell-autonomous defects within the germ cells or defects in the surrounding soma still remains unclear. Recently, miR-430, which has the same seed sequence as many members of the miR-290 family, was implicated in PGC migration in the zebrafish (Staton et al., 2011). Interestingly, loss of miR-430 led to defective migration due to misexpression of chemokines in the surrounding soma. The *mir-290-295* locus is expressed in migrating PGCs (Hayashi et al., 2008). Furthermore, the *mir-290-295* primary transcript is expressed in the early (E6.5) embryo and its expression decreases by E10.5 (Fig. 2.1A). Currently it is not known whether or not miR-290 miRNAs are present in the tissues through which the PGCs must migrate. Therefore, we cannot rule out the possibility that a subtle defect in the surrounding soma might be the cause of the ectopic germ cells.

Little is known about the genes involved (both the in germ cells themselves and the soma) in the early stages (E7.5-E8.5) of germ cell migration. Although on the basis of misexpression studies *Ifitm1 (fragilis)* was thought to play a role in movement of the primordial germ cells from the epiblast into the endoderm (Tanaka et al., 2005), an *Iftim1*

homozygous knockout showed no defects in germ-cell localization (Lange et al., 2008). To the best of our knowledge, the *mir-290-295* deletion represents the first germ-cell mutant where PGCs mislocalize to the ventral surface of the embryo near the developing hindlimbs. Thus, it will be of interest to understand the molecular mechanisms of how the *mir-290-295* cluster is involved in the early stages of germ-cell migration.

Our data cannot exclude the possibility that the migration defect observed is an indirect effect of improper germ-cell specification. We do not know whether the same number of germ cells is allocated in *mir-290-295-/-* and wild-type animals. Further experiments beyond the scope of this work are required to address these questions.

The ability of the *mir-290-295-/-* males to recover from the early germ-cell loss likely is a result of the extended proliferative lifespan of male germ cells rather than a direct consequence of *mir-290-295* deficiency. Recovery of male germ cells after embryonic sex-neutral depletion has been previously reported (Luoh et al., 1997; Lu and Bishop, 2003) and is therefore not unique to the *mir-290-295* mutant. Male germ cells undergo mitotic arrest around E13.5 but then resume mitosis a few days after birth (Lu and Bishop, 2003). In contrast, female germ cells enter meiosis around E13.5, thereby establishing the total oocyte pool for adult life (Bristol-Gould et al., 2006). When oocytes leave the primordial follicle pool to undergo maturation and/or death they are not able to be replaced. The extra proliferative time in the males allows for additional clonal expansion of the few surviving *mir-290-295-/-* gonocytes which results in enough germ cells for the *mir-290-295-/-* males to maintain fertility.

***mir-290-295* deficiency confers incompletely penetrant phenotypes**

The phenotypes conferred by *mir-290-295* deficiency are characterized by variable expressivity and incomplete penetrance. For example, three-quarters of knockouts die

before birth, with the remainder surviving until adulthood. The mixed background of the mice used in this study (129/C57BL6) may contribute to the incomplete penetrance of the embryonic lethality. However, given what is known about miRNAs, the partially penetrant embryonic lethality might also be explained by the function of the *mir-290* cluster itself. MicroRNAs have been shown to confer robustness to developmental systems (Hornstein and Shomron, 2006; Tsang et al., 2007; Martinez et al., 2008; Li et al., 2009; Herranz and Cohen, 2010). Because of its position in a genetic regulatory network, a microRNA can modulate expression of its target genes, thereby buffering random fluctuations in gene expression. It was recently shown that random fluctuations in gene expression can result in an incompletely penetrant phenotype when a certain level of gene expression is required to pass a threshold in order to cause an outcome (Raj et al., 2010). Consistent with this, deletions of various microRNAs have been shown to confer partially penetrant phenotypes (Li et al., 2006; Zhao et al., 2007; Fish et al., 2008; Kuhnert et al., 2008). Taken together these data lead us to speculate that the loss of *mir-290-295* expression might cause fluctuations in gene expression patterns. Those mutants with gene expression patterns that differ greatly from wild-type would not survive while mutants with gene expression patterns close to their wild-type counterparts would be able to develop normally during this period. Presumably after this time window, the role of *mir-290-295* becomes less critical. Therefore any mutants surviving this time period also survive to adulthood.

Materials and Methods

RT-PCR

RNA samples were isolated by homogenizing tissue or cells in Trizol (Invitrogen, Carlsbad, CA, USA) following manufacturers suggested protocol. Five micrograms of total RNA was DNase I treated using the DNA-Free RNA Kit (Zymo Research, Orange, CA, USA). One microgram of DNase I-treated RNA was reverse transcribed using a First Strand Synthesis

Kit (Invitrogen). PCR was performed using 1/80 of the reverse transcription reaction. The following primer sequences were used to determine *pri-mir-290-295* expression: 5'-GAACCTCACGGGAAGTGACC-3' (forward primer) and 5'-TGCCCACAGGAGAGACTCAA-3' (reverse primer).

Northern blot analysis

RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA) following manufacturer's instructions. 30 µg of total RNA was electrophoresed for 45 minutes at 35 W, and semidry transferred to Hybond-NX nylon membrane (Amersham Biosciences, Piscataway, NJ, USA) at 18 V for 1.5 hours at 4ºC. RNA was cross-linked and incubated with LNA probe as previously described (Valoczi et al., 2004; Pall et al., 2007). After washing, membranes were exposed to a phosphorimager screen for 1-3 days depending on the probe. Probes were synthesized by IDT (San Diego, CA, USA): (1) miR-17, C+TAC+CTG+CAC+TGT+AAG+CAC+TTT+G, (2) miR-295, A+GAC+TCA+AAA+GTA+GTA+GCA+CTT+T, and (3) tRNA glu, TGGAGGTTCCACCGAGAT, where + indicates that the following nucleotide is a LNA.

Generation of *mir-290-295 -/-* mice

Mice deficient for *mir-290-295* were generated by targeted disruption of the endogenous *mir-290-295* locus via homologous recombination in ES cells. Upstream and downstream arms were PCR amplified from RPCI-23-222D1 BAC DNA, resulting in a construct where 2.1 kb of the *mir-290-295* locus (including the mature miRNA sequences) was replaced by a 1.6 kb neomycin resistance selection cassette (Fig. 2.2A). This targeting construct was electroporated into V6.5 ES cells that were then subjected to selection with G418. After 10 days of selection, G418 resistant clones were analyzed by Southern blotting with external probes. Clones exhibiting correct targeting were injected into B6D2F2 recipient blastocysts for subsequent chimera generation. For this study, mice were maintained on a 129S4 x C57BL/6 mixed genetic background. The Committee on Animal Care at the Massachusetts Institute of Technology approved all experiments involving mice.

ES cell culture, manipulations and derivation

ES cells were cultured on irradiated mouse embryonic fibroblasts in DMEM containing 10% fetal bovine serum, leukemia inhibiting factor (LIF), penicillin/streptomycin, L-glutamine and

nonessential amino acids. For ES cell derivation, zonae pellucidae were removed using acidic Tyrode's (AT) solution and blastocysts were explanted on irradiated feeders in ES medium supplemented with MEK1 inhibitor (PD98059, Cell Signaling Technology, Danvers, MA, USA).

Antibodies, histological and immunohistochemical analysis of adult and embryonic gonads

For immunohistochemistry and histology, gonads were fixed in either 4% paraformaldehyde or Bouin's solution overnight, embedded in paraffin and sectioned. For immunohistochemistry, slides were de-waxed, rehydrated and steamed in 10 mM Sodium Citrate buffer, pH 6.0 for 24 minutes. Rabbit polyclonal anti-MVH (Abcam, Cambridge, MA, USA) was used at a dilution of 1/200, mouse monoclonal anti-PCNA (Abcam, Cambridge, MA, USA) was used at a dilution of 1/1000, polyclonal rabbit anti-cleaved-caspase-3 (Cell Signaling Technology, Beverly, MA, USA) was used at a dilution of 1/200, rat monoclonal anti-Ki-67 (Dako, Carpinteria, CA, USA) was used at a dilution of 1/200, mouse monoclonal SSEA-1 (Developmental Studies Hybridoma Bank, Iowa, USA) was used at a dilution of 1/20, and rat anti-GCNA1 (a gift from George Enders, University of Kansas Medical Center, Kansas City, KS) was used as undiluted supernatant. Images were captured with a Nikon E800 upright microscope (MVH staining, Figure 2.5) or a PerkinElmer Ultraview Spinning Disk Confocal Microscope (Figures 2.7, 2.S3, 2.S4).

To determine the germ-cell density in control and mutant E13.5 gonads the following methods were used. For E13.5 males, the number of MVH-positive cells in each tubule was counted for at least three non-consecutive histological sections for each gonad from each animal. The analysis program Image J (Abramoff et al., 2004) was used to determine the total relative tubule area for each section. The total number of MVH-positive germ cells counted for all sections was then divided by the total tubule area for all sections to calculate a relative germ- cell density for each testis. The two germ-cell densities for each testis were then averaged to obtain the average germ-cell density for each E13.5 male. For females, the total number of MVH-positive cells in at least two non-consecutive histological sections was counted and Image J was used to calculate the relative area of each ovarian section. Then the total number of MVH-positive germ cells counted for all sections for each ovary was divided by the total relative area. The two germ-cell densities for each ovary were then averaged to obtain the average germ-cell density for each E13.5 female.

For E12.5 the middle longitudinal section for each gonad was used for counting.

Whole-mount alkaline phosphatase staining

Alkaline phosphatase staining was performed according to procedure outlined in Hara et al (Hara et al., 2009). Briefly, E9.5 embryos were dissected into PBS, fixed 6 hours - overnight at 4°C in 4% paraformaldehyde then washed for 12 hours in TBS-T. The samples were then stored in 70% ethanol for at least 3 days. After several washes in NTMT buffer (100 mM NaCl, 100 mM Tris-HCl (pH 9.5), 50 mM $MgCl_2$, 0.1% Tween-20) the samples were stained with BCIP-NBT (Roche Applied Science, Indianapolis, IN, USA). The hindgut and developing hindlimb areas were dissected away from the embryo and flattened under a coverslip to facilitate counting of alkaline phosphatase-positive cells.

Genotyping of *mir-290-295* embryos

Midday of the day of the vaginal plug was considered as 0.5 dpc in the timing of embryo collection. Embryos were dissected from the decidua at various time points during development. Blastocysts were flushed from the ovaries at 3.5 dpc and dissociated in 30µl lysis buffer (50 mM Tris pH 8.8, 1 mM EDTA, 0.5% Tween20, 200 µg/ml Proteinase K) for 4 hours at 55°C. Samples were then heated to 95°C for 10 minutes to inactivate Proteinase K. Successful genotyping of blastocysts required nested PCRs. For first round PCR, 5 µl of lysed blastocyst was used and then 2 µl of first round PCR reaction was used for the second round of amplification. The following primers were used: endogenous allele first round PCR: 5'-CTACAATGCACCTGGACTCA-3' (forward) and 5'-AGAGGCGAAAGTAGATCCAG-3'(reverse), endogenous allele second round PCR: 5'-CGGTTTGGCTGGGTTTACTA-3' (forward) and 5'-AACGACCACCTCAGTTACCG-3' (reverse); targeted allele first round PCR: 5'-AGAGGCCACTTGTGTAGCGC-3 (forward) and 5'-AGAGGCGAAAGTAGATCCAG-3'(reverse), targeted allele second round PCR: 5'-CGGTTTGGCTGGGTTTACTA-3' (forward) and 5'-CAGACTGCCTTGGGAAAAGT-3' (reverse). For embryos E7.5 and older one round of amplification was sufficient for genotyping.

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Tables

Table 2.1: *mir-290-295* deficiency results in partially penetrant embryonic lethality. Embryos from heterozygous matings were dissected at the specified timepoints and genotyped. The middle column lists the numbers of wild-type, heterozygous (*mir-290-295+/-*), and homozygous knockout (*mir-290-295-/-*) embryos observed at each timepoint. The percentages in parentheses are the percentages of total embryos that were found to be homozygous knockout at each particular timepoint. For example, at E3.5, 27% (32 out of 117) of progeny from heterozygous intercrosses were *mir-290-295-/-*. Reabsorbed embryos refer to embryos which were too disintegrated to separate from the maternal tissue. These reabsorbed embryos were not genotyped. *p<0.01, chi squared test, **p<0.001, chi squared test.

	wild-type: het: knockout (% knockout)	reabsorbed embryos
E3.5	29 : 56 : 32 **(27%)**	----------
E7.5	2 : 4 : 2 **(25%)**	0
E8.5	7 : 21 : 13 **(32%)**	0
E9.5	18 : 30 : 21 **(30%)**	0
E10.5	14 : 36 : 12 **(19%)**	1
E11.5	10 : 19 : 6 **(17%)**	4
E13.5	16 : 26 : 10 **(19%)**	10
E18.5	8 : 35 : 3 **(7%)***	8
Postnatal (4 weeks)	168 : 252 : 32 **(7%)****	----------

Table 2.2: *mir-290-295*-deficient embryos display defects at midgestation stages. *mir-290-295-/-* embryos analyzed in this table are progeny from both *mir-290-295+/-* intercrosses and *mir-290-295+/-* × *mir-290-295-/-* crosses. "Abnormal" refers to embryos that were either observed outside the yolk sac or were developmentally delayed compared to wild-type littermates. In the last column the percentages in parentheses are the percentages of total *mir-290-295-/-* embryos observed that were abnormal or reabsorbed. For example, at E8.5, 64% (16 out of 25) embryos were either abnormal or reabsorbed.

	Total number of *mir-290-295-/-* embryos observed	Number of abnormal or reabsorbed *mir-290-295-/-* observed (% abnormal)
E3.5	32	0 **(0%)**
E8.5	25	16 **(64%)**
E9.5	39	26 **(66%)**
E10.5	22	14 **(64%)**
E11.5	10	5 **(50%)**
E13.5	21	11 **(52%)**
E18.5	11	8 **(73%)**

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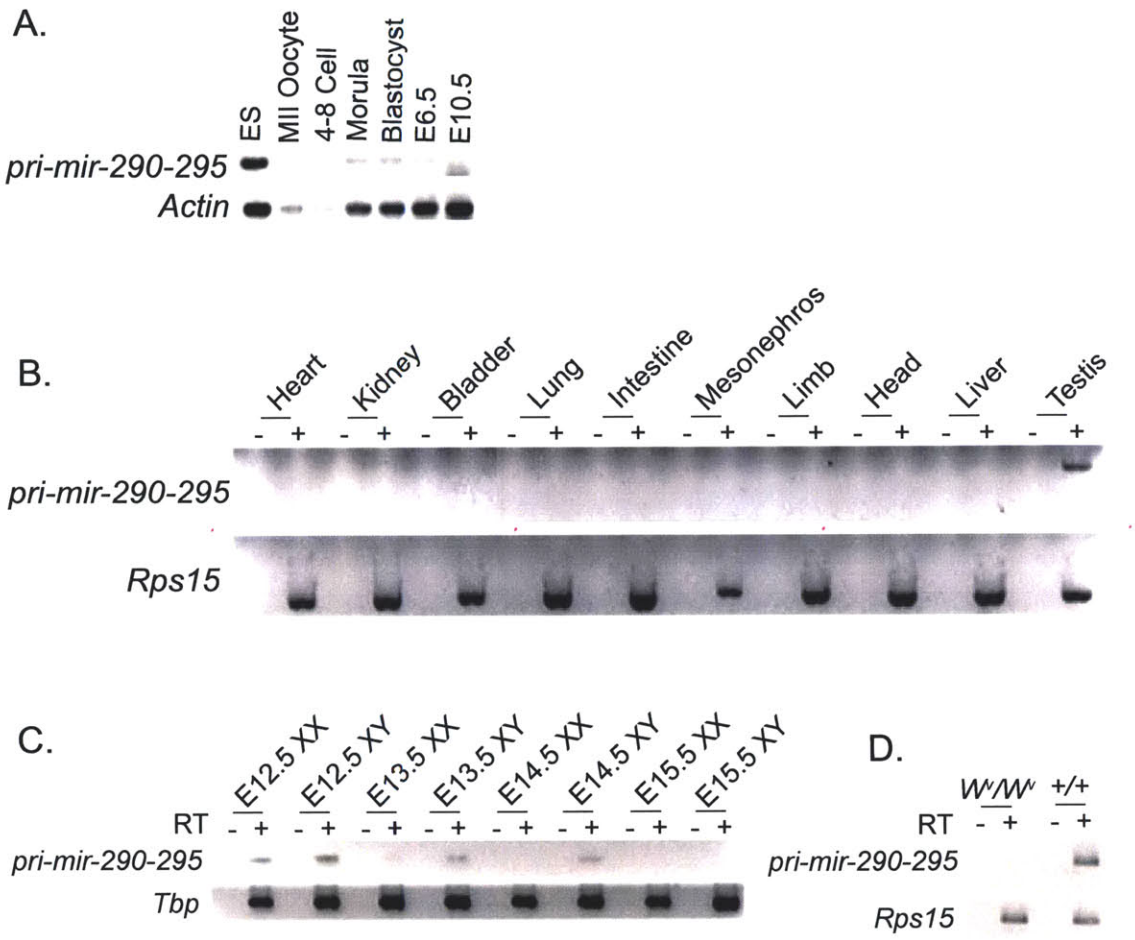


Figure 2.1: *mir-290-295* is expressed in the early embryo and in germ cells. (A) RT-PCR of *pri-mir-290-295* and *Actin* control in early embryos. (B) RT-PCR of p*ri-mir-290-295* and *Rps15* control in a tissue panel from **E14.5** embryos. (C) RT-PCR of *pri-mir-290-295* and *Tbp* control in E12.5-E15.5 embryonic gonads from males and females as indicated. (D) RT-PCR of *pri-mir-290-295* and *Rps15* control in E14.5 testis isolated from wild-type and homozygous *c-kit* (W^v) mutants.

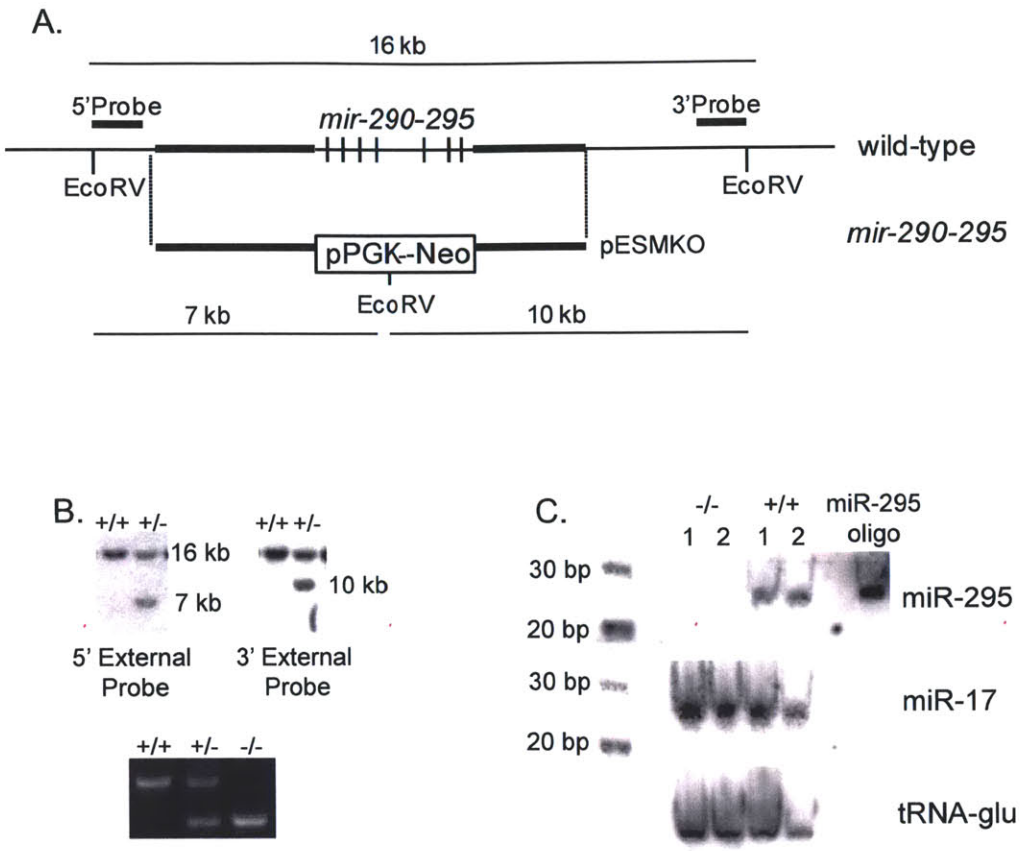


Figure 2.2: Targeted disruption of the *mir-290-295* locus. (A) Targeting strategy for generation of the *mir-290-295* allele. (B) Southern blot and PCR confirmation of correct targeting of the *mir-290-295* locus. (C) Northern blot validation of *mir-290-295* targeting. Two *mir-290-295-/-* ES cell lines (-/- 1 and 2) and two wild-type ES cell lines (+/+ 1 and 2) were derived and used to verify correct targeting of the *mir-290-295* locus.

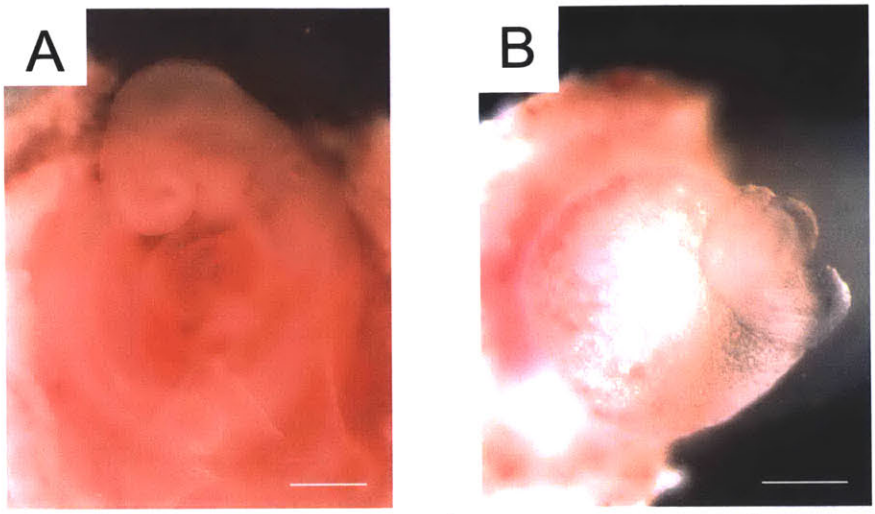


Figure 2.3: Some *mir-290-295-/-* embryos were abnormal and observed outside of the yolk sac. (A) Wild-type E9.5 embryo. (B) *mir-290-295-/-* E9.5 embryo located outside of the yolk sac. Scale bars, 500μm.

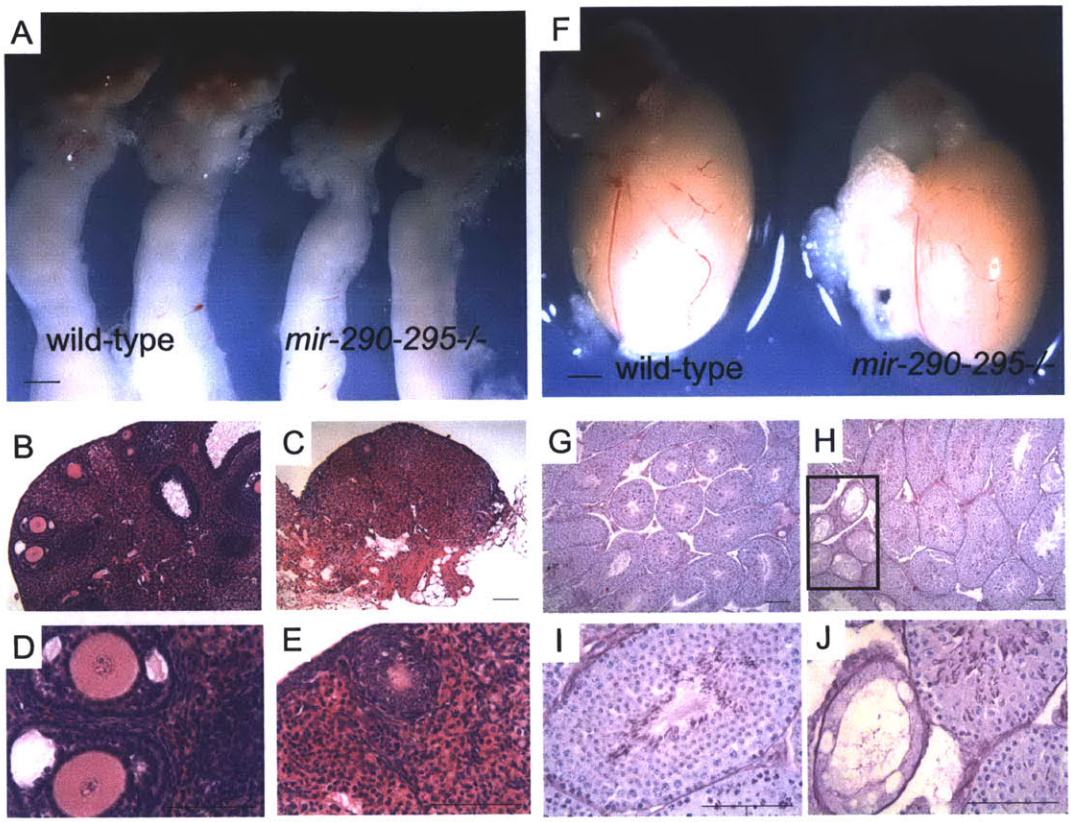


Figure 2.4: Adult male *mir-290-295-/-* testes show reduced germ cells compared to wild-type testes whereas adult female *mir-290-295-/-* ovaries are atrophied. (A) Ovaries and reproductive tracts from 8-week old wild-type and *mir-290-295-/-* females. Note the smaller, atrophied ovaries of *mir-290-295-/-* females. Scale bar, 1mm. (B-E) Hematoxylin and eosin-stained ovary sections from adult wild-type (B&D) and *mir-290-295-/-* (C&E) animals. D and E are enlargements of B and C, respectively. Scale bars, 100µm. (F) Testes from 8-week old wild-type and *mir-290-295-/-* males. Note that the smaller testes size of the knockout male is at least partially due to the smaller body weight of the knockout animal. Scale bar, 1mm. (G-J) Periodic acid-Schiff (PAS)-stained testes sections from adult wild-type (G&I) and *mir-290-295-/-* (H&J) males. I and J are enlargements of G and H, respectively. The black rectangle in H highlights the area with empty seminiferous tubules. Scale bars, 100µm. Please refer to Figure 2.S1 for images of early postnatal testes and ovaries.

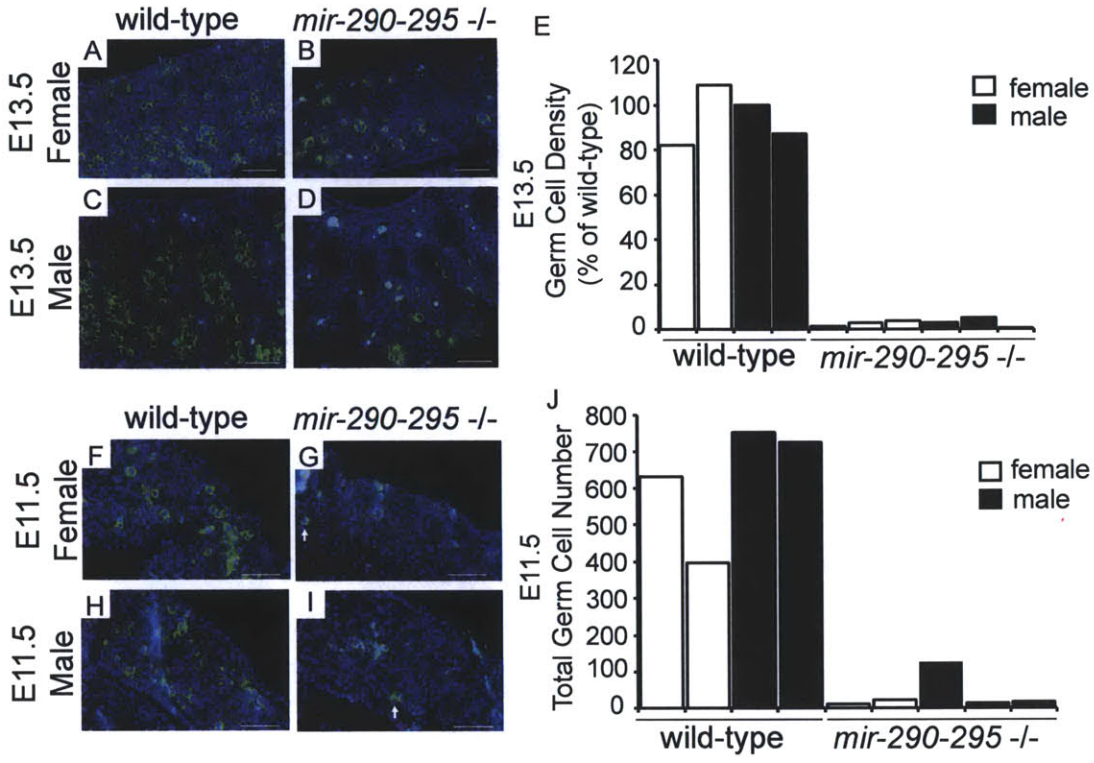


Figure 2.5: Both male and female *mir-290-295-/-* embryonic gonads show reduced germ cell numbers as early as E11.5. (A-D) Representative MVH(green)-stained sections of E13.5 ovaries (A&B) and testes (C&D) from wild-type (A&C) and *mir-290-295-/-* (B&D) embryos. Scale bars, 50μm. (E) Germ-cell density (germ cells/area of gonadal section for ovaries and germ cell/testis cord area for testes) as determined by MVH staining. Each bar represents a single embryo with black bars indicating male embryos and white bars indicating female embryos. (F-I) Representative MVH(green)-stained sections of E11.5 genital ridges from wild-type (F&H) and *mir-290-295-/-* (G&I) embryos. Scale bars, 50μm. White arrows point to germ cells in G and I. The additional green cells in I are blood cells. (J) Total germ cell numbers in E11.5 gonads as determined by serial sectioning and MVH staining. Each bar represents a single embryo with black bars indicating male embryos and white bars indicating female embyros.

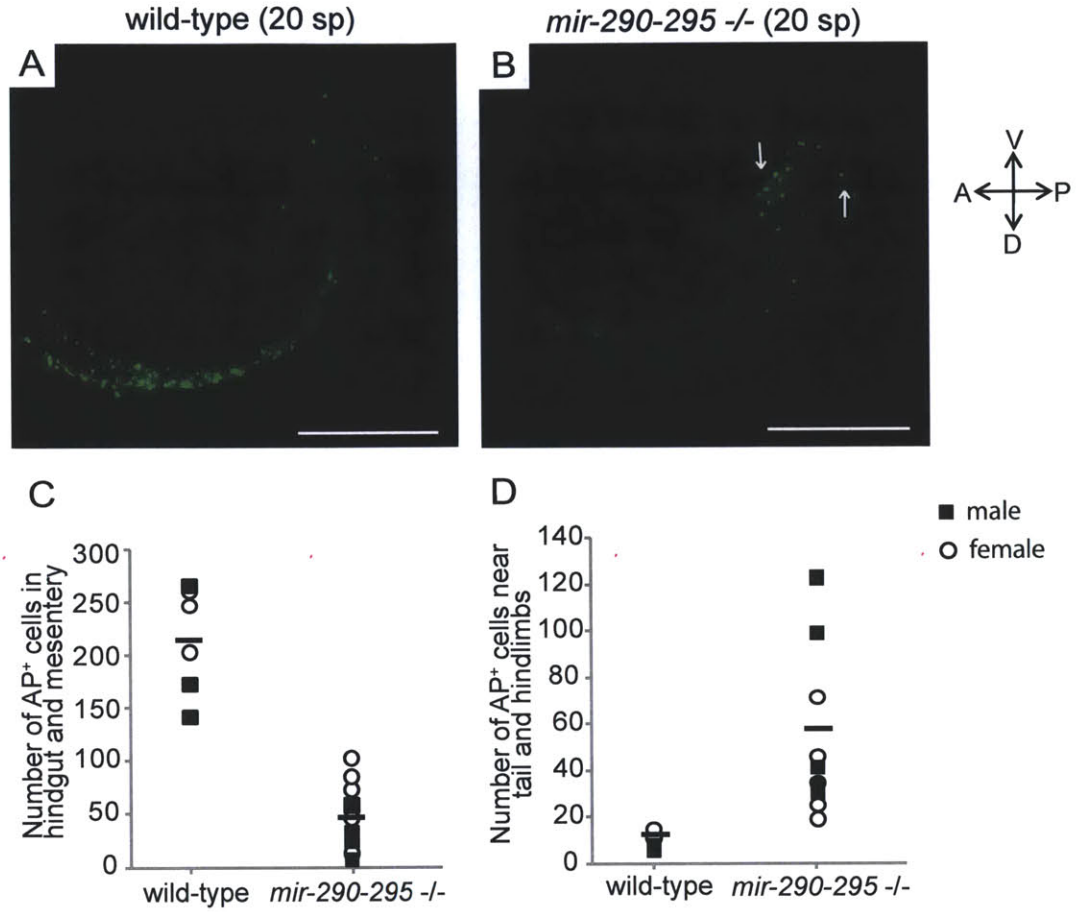
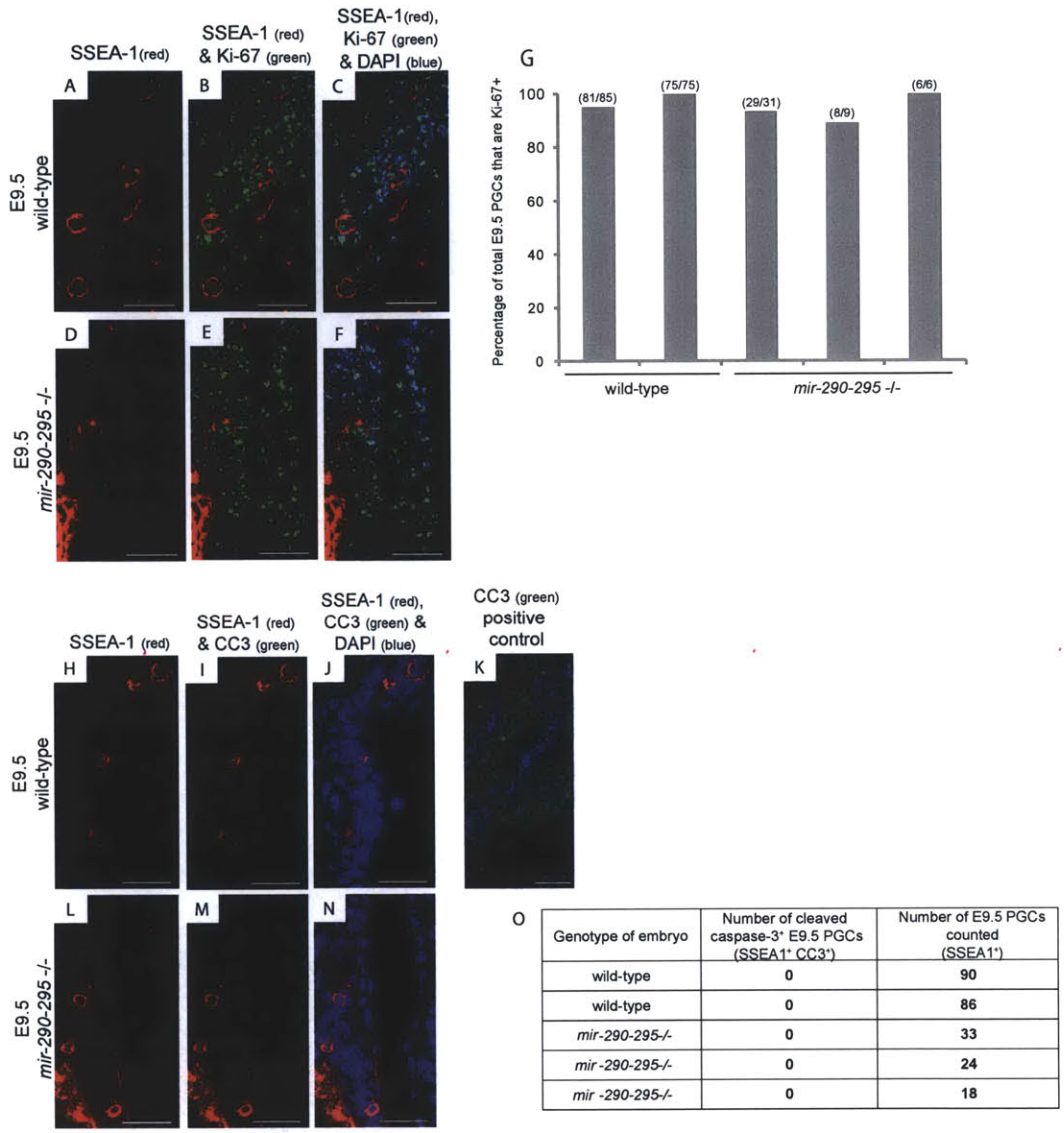


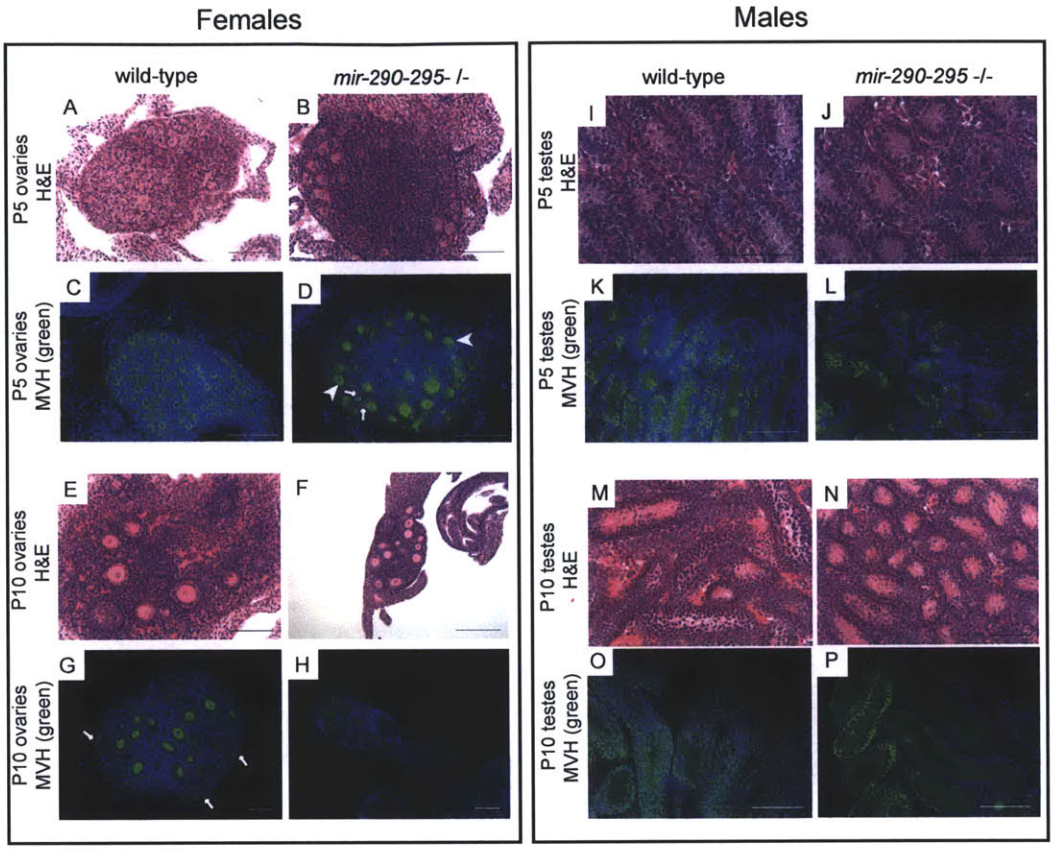
Figure 2.6: Primordial germ cells (PGCs) are mislocalized in *mir-290-295-/-* embryos. (A) Image of E9.5 *Oct4-GFP* wild-type embryo showing the migration of *Oct4-GFP* PGCs along the hindgut. (B) Image of E9.5 *Oct4-GFP mir-290-295-/-* embryo. Note that the majority of *Oct4-GFP* positive PGCs are not in the hindgut, but rather are mislocalized near the tail (white arrows). Scale bars, 1mm. The numbers in parentheses refer to the numbers of somite pairs in each embryo. (C) Number of PGCs, as determined by alkaline phosphatase staining and manual counting, in the hindgut and mesentery of E9.5 embryos. Black bars indicate the average number of PGCs for each genotype. (D) Number of PGCs, as determined by alkaline phosphatase staining and manual counting, near the hindlimb and base of tail, in E9.5 embryos. Black bars indicate the average number of PGCs for each genotype.



O

Genotype of embryo	Number of cleaved caspase-3⁺ E9.5 PGCs (SSEA1⁺ CC3⁺)	Number of E9.5 PGCs counted (SSEA1⁺)
wild-type	0	90
wild-type	0	86
mir-290-295-/-	0	33
mir -290-295-/-	0	24
mir -290-295-/-	0	18

Figure 2.7: *mir-290-295-/-* E9.5 PGCs do not undergo premature cell-cycle arrest or apoptosis. (A-F) Representative sections from E9.5 wild-type (A-C) and *mir-290-295-/-* (D-F) embryos stained for SSEA-1, Ki-67, and DAPI. Scale bars, 25μm. (G) Percentage of total PGCs that are Ki-67-positive. Numbers in parentheses show the actual number of Ki-67-positive PGCs (SSEA1+ Ki-67+ cells) over the total number of PGCs counted for that embryo (SSEA-1+ cells). (H-N) Representative sections from E9.5 wild-type (H-J) and *mir-290-295-/-* (L-N) embryos stained for SSEA-1, cleaved-caspase-3, and DAPI. Scale bars, 25μm. (K) Section of a dying embryo stained for cleaved-caspase-3 and DAPI in order to prove the validity of the antibody and staining procedure. (O) Table outlining the number of cleaved-caspase-3-positive PGCs observed in wild-type and *mir-290-295-/-* E9.5 embryos. Note that no PGCs in either wild-type or *mir-290-295-/-* embryos were positive for cleaved-caspase-3.



Supplemental Figure 2.S1: Both male and female early postnatal (P5 and P10) *mir-290-295-/-* gonads show germ cell depletion. (A&B) Hematoxylin and eosin-stained sections from wild-type (A) and *mir-290-295-/-* (B) P5 ovaries. (C&D) MVH-stained sections from wild-type (C) and *mir-290-295-/-* (D) P5 follicles. Arrows in D point to primordial follicles while arrowheads point to growing follicles. Note that *mir-290-295-/-* P5 ovaries have fewer primordial follicles than wild-type ovaries. (E&F) Hematoxylin and eosin-stained sections from wild-type (E) and *mir-290-295-/-* (F) P10 ovaries. (G&H) MVH-stained sections from wild-type (G) and *mir-290-295-/-* (H) P10 ovaries. Arrows point to the primordial follicle pool. Note the absence of the primordial follicle pool in the mutant. (I&J) Hematoxylin and eosin-stained sections from wild-type (I) and *mir-290-295-/-* (J) P5 testes. (K&L) MVH-stained sections from wild-type (K) and *mir-290-295-/-* (L) P5 testes. (M&N) Hematoxylin and eosin-stained sections from wild-type (M) and *mir-290-295-/-* (N) P10 testes. (O&P) MVH-stained sections from wild-type (O) and *mir-290-295-/-* (P) P10 testes. Note that both P5 and P10 *mir-290-295-/-* testes have fewer germ cells than wild-type testes. Scale bars, 100μm.

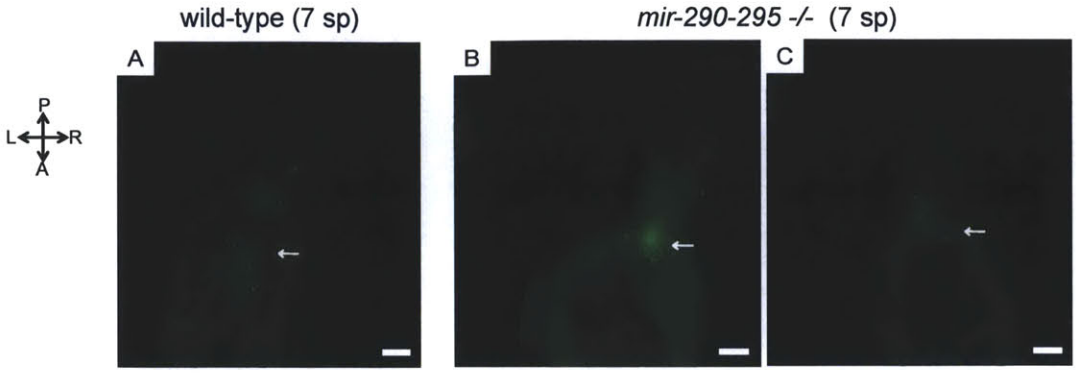


Figure 2.S2: *mir-290-295-/-* PGCs are delayed in entering the hindgut. (A) Image of E8.5 *Oct4-GFP* wild-type embryo showing that PGCs have successfully entered the hindgut (white arrow). (B&C) Images of E8.5 *Oct4-GFP mir-290-295-/-* embryos. Note that in both mutant embryos *Oct4-GFP*-positive PGCs appear stuck at the base of the allantois and have not yet entered the hindgut (white arrows). The numbers in parentheses refer to the number of somite pairs present in each embryo. Scale bars, 100 μm.

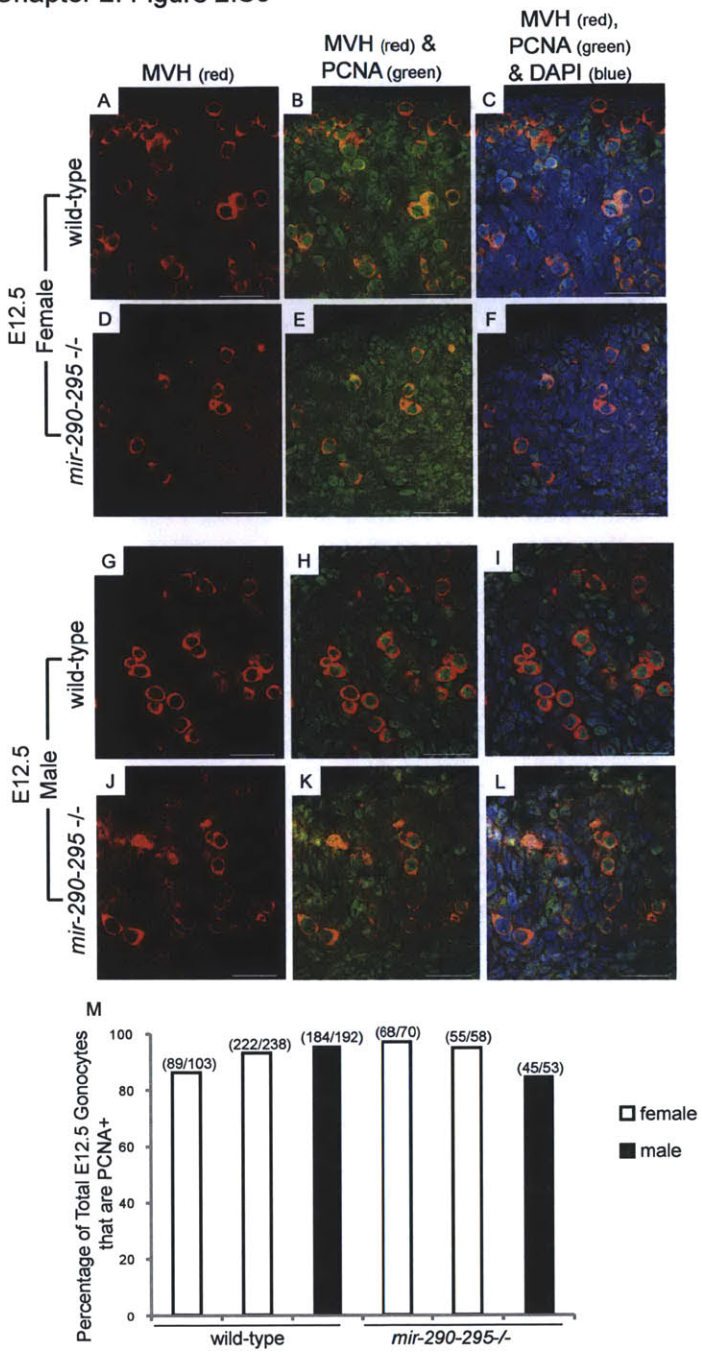
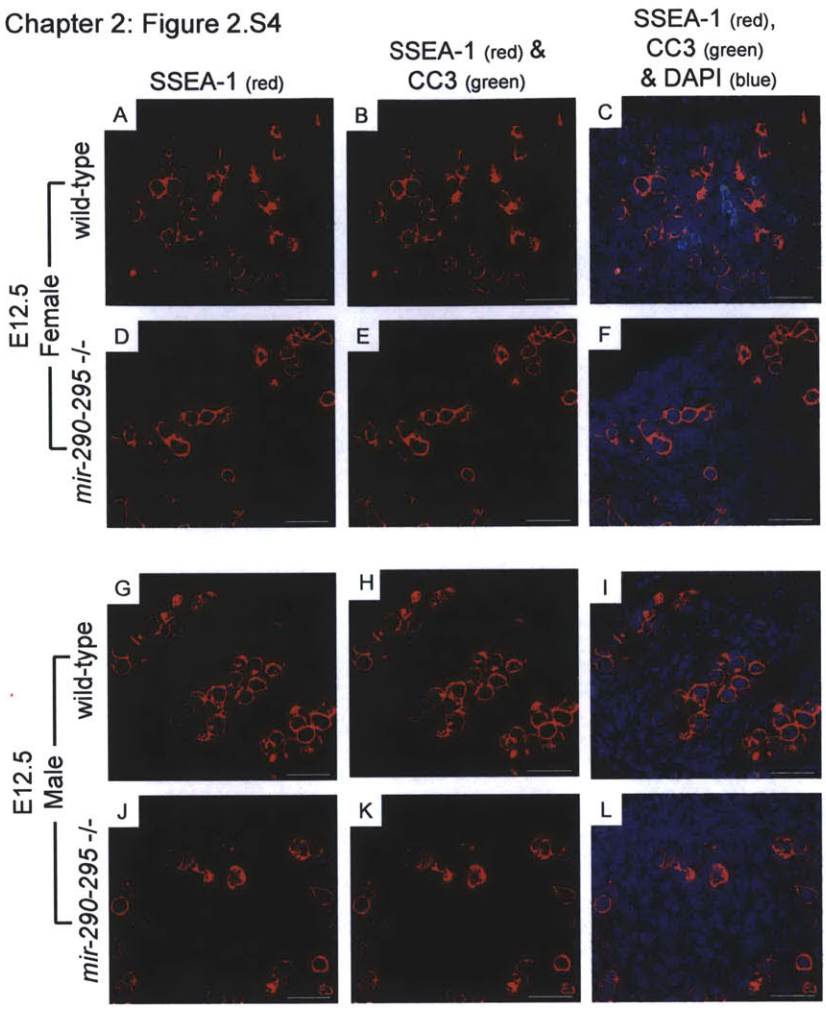


Figure 2.S3: *mir-290-295-/-* gonocytes do not undergo premature cell-cycle arrest. (A-F) Representative sections of female E12.5 wild-type (A-C) and *mir-290-295-/-* (D-F) gonads stained for MVH, PCNA, and DAPI. Scale bars, 25μm. (G-L) Representative sections of male E12.5 wild-type (G-I) and *mir-290-295-/-* (J-L) gonads stained for MVH, PCNA, and DAPI. Scale bars, 25μm. (M) Percentage of total gonocytes that are PCNA+. Numbers in parentheses show the actual number of PCNA+ PGCs (PCNA+ MVH+ cells) over the total number of PGCs counted for the mid-gonadal section for that embryo (MVH+ cells).



M

Genotype of embryo	Number of cleaved caspase-3⁺ E12.5 gonocytes (SSEA1⁺ CC3⁺)	Number of E12.5 gonocytes counted (SSEA1⁺)
Females: wild-type	**0**	**159**
wild-type	**0**	**88**
mir-290-295 -/-	**0**	**84**
mir-290-295 -/-	**0**	**84**
Males: wild-type	**0**	**165**
mir-290-295 -/-	**0**	**93**

Figure 2.S4: *mir-290-295-/-* gonocytes do not undergo apoptosis. (A-F) Representative section of female E12.5 wild-type (A-C) and *mir-290-295-/-* (D-F) gonads stained for SSEA-1, cleaved-caspase-3, and DAPI. Scale bars, 25μm. (G-L) Representative section of male E12.5 wild-type (G-I) and *mir-290-295-/-* (J-L) gonads stained for SSEA-1, cleaved-caspase-3, and DAPI. Scale bars, 25μm. (M) Table outlining the number of cleaved-caspase-3+ gonocytes observed in a mid-gonadal section from either wild-type or *mir-290-295-/-* E12.5 gonads.

Chapter 3

Conclusions

Introduction

mir-290-295 deficiency confers two main phenotypes –partially penetrant embryonic lethality and early germ-cell loss. At this point, the detailed mechanistic explanations behind the *mir-290-295* loss-of-function phenotypes are not known. It is not even clear what causes the early embryonic lethality. Not all *mir-290-295 -/-* embryos exhibit the exact same abnormalities – some are malformed and located outside of the yolk sac at E9.5 and some are merely developmentally delayed by several hours. Therefore, is the early embryonic lethality the result of one specific defect or do multiple defects contribute to this phenotype? We have also not ruled out the possibility that both the embryonic lethality and the germ-cell loss originate from one defect caused by loss of miR-290 family miRNAs.

Searching for the mechanisms behind the *mir-290-295* loss-of-function phenotype requires careful examination of the earliest developmental defects and earliest germ-cell-specific defects in these mutants. Later abnormalities, no matter how unlikely it may seem, could be the result of these earliest defects. Because each miRNA can have hundreds of targets and the miR-290 family contains at least seven miRNAs, there are thousands of predicted targets for this cluster. Detailed study of these earliest phenotypes would provide additional information which would help to select the physiologically relevant targets from this long list. This conclusion will consider the possible origins of the developmental delay, out-of-yolk-sac phenotype, and germ-cell mislocalization conferred by loss of *mir-290-295-/-*. It will conclude with a discussion of how the partial penetrance observed in *mir-290-295-/-* animals might be explained by the theory that miRNAs can promote robustness.

All miR-290 family members are not the same

The knockout approach used in this thesis treats the miRNAs derived from the *mir-290-295* cluster as a unit. However, as mentioned in Chapter 1, studies have unearthed differences between the various miRNAs encoded by this cluster (Wang et al., 2008; Ciaudo et al., 2009; Judson et al., 2009; Spruce et al., 2010; Zheng et al., 2011). Many miRNAs encoded by the *mir-290-295* cluster have the same AAGUGC seed sequence (miR-290-3p, miR-291a-3p, miR-291b-3p, miR-292-3p, miR-294, miR-295) while others do not (miR-290-5p, miR-291a-5p, miR-291b-5p, miR-292-5p, miR-293). Because the seed sequence is not the only factor determining target recognition, it is possible that even the miR-290 family miRNAs with the same seed sequence regulate different targets (Grimson et al., 2007). Experimental evidence further supports the idea that not all miR-290 miRNAs are alike. For example, miR-291a-3p, miR-291b-3p, miR-294, and miR-295 are able to rescue the cell-cycle defects in *Dgcr8*-deficient ES cells while other miRNAs encoded by the *mir-290* cluster, such as miR-290-3p and miR-293 are not.

However, the *mir-290-295-/-* mouse line does provide a way to test the functions of individual miRNAs within the family. Reintroduction of specific miRNAs into this *mir-290-295-/-* background could determine which miRNA or which combination of miRNAs rescue which phenotypes.

Potential causes of developmental delay conferred by *mir-290-295* deficiency

The greatest difference between wild-type and *mir-290-295-/-* embryos was the developmental delay, manifested by the mutant embryos. This developmental delay was assessed mainly by somite number but also by various morphological landmarks characteristic of each developmental stage. Even at the earliest timepoint examined, E7.5,

the mutants were delayed compared to their wild-type siblings. This delay persisted throughout all developmental timepoints monitored, E8.5, E9.5, E10.5, E11.5 and E13.5.

Failure to generate a critical cell mass early in development can lead to developmental delay. Mouse embryos with a deficient number of epiblast cells, caused either by removal of blastomeres or apoptosis induced by acute exposure to a DNA-damaging agent, have been shown to reschedule gastrulation and other morphogenetic events until they have reached a critical mass (Snow and Tam, 1979; Rands, 1986; Power and Tam, 1993). Impressively, these embryos are able to compensate for the early cellular loss both by accelerating cellular proliferation and extending the proliferative phase beyond its normal duration in untreated embryos. The increase in proliferation rate is accomplished through shortening of the S and G2 phases of the cell cycle (Power and Tam, 1993). Although organogenesis proceeds via a slightly different schedule in the treated embryos, by late preimplantation development, these treated embryos display properly formed organs and similar numbers of somites as their untreated counterparts. These data show that the mouse embryo can be reduced to 10% of its size before organogenesis but be nearly normal before that stage is complete (Snow and Tam, 1979).

If the developmental delay observed in *mir-290-295-/-* embryos is due to a low number of epiblast cells, what might be the cause of these reduced cell numbers? Slower cellular proliferation or apoptosis could both result in a decreased number of epiblast cells. Interestingly, *Dicer-/-* embryos display increased apoptosis in the epiblast (Spruce et al., 2010). This phenomenon was correlated with increased amounts of the proapoptotic protein Bim. Since miR-17 family members are known to target *Bim* mRNAs, it was hypothesized that these miRNAs normally prevent apoptosis in the epiblast. However, miR-290-295 miRNAs are also expressed in the gastrulating embryo and they have been shown to protect ES cells from genotoxic stress by downregulating proapoptotic proteins, Caspase 2 and Ei24

(Zheng et al., 2011). Therefore it would be valuable to test whether *mir-290-295-/-* epiblasts show increased apoptosis compared to their wild-type counterparts.

MiR-290 miRNAs also work to promote the rapid cell cycle characteristic of ES cells. Members of the miR-290 family are able to rescue the proliferation defect in *Dgcr8-/-* mice (Wang et al., 2008). miR-290 family members accomplish this by targeting inhibitors of cyclinE-Cdk2. In addition to ES cells, early mouse embryos also have fast cell cycles which could potentially be regulated by *mir-290-295*. Between E4.5 and E6.0, the average cell-cycle time of early embryonic cells is 10h and during E6.5-7 generation times can be as short as 4.4hr (Power and Tam, 1993; White and Dalton, 2005). In fact, the cell cycles of ES cells and early embryos are believed to be so similar that ES cell are often used as a substitute to study factors controlling the early embryonic cell cycle (White and Dalton, 2005). A slower cell cycle in the early mutant embryo could certainly result in fewer cells. Furthermore, if increased apoptosis did occur in early *mir-290-295-/-* embryos, a slower cell cycle would definitely impair the ability of *mir-290-295-/-* embryos to catch up to their wild-type siblings. Cell-cycle analysis of cells from early *mir-290-295-/-* embryos would help to address these possibilities.

Potential causes of out-of-yolk-sac phenotype conferred by mir-290-295 deficiency

A portion of *mir-290-295-/-* embryos were malformed and found either partially or completely outside of the yolk sac at E8.5 and E9.5. The phenomenon of malformed embryos outside of the yolk sac has been observed for mutants of several genes involved in patterning the embryo during and after gastrulation. These mutants include the *Nodal* (hypomorph) (Lowe

et al., 2001), *activin receptor (lf)* mutant (Song et al., 1999), *Hnf3B(lf)* mutant (Dufort et al., 1998), *Foxh1(lf)* mutant (Norris et al., 2002) , and *axin* (lf) mutant (Zeng et al., 1997) . Many of these mutants have defects in distal visceral endoderm (DVE) translocation (Fig.3.1). The distal visceral endoderm (DVE) serves as a signaling center primarily by secreting *Nodal* antagonists such as LEFTY1 and *Wnt* antagonists such as CER1 (Cerberus like 1) (Arnold and Robertson, 2009). Around E5.5, the distal visceral endoderm translocates to the future anterior side of the embryo and becomes known as the anterior visceral endoderm (AVE). At this time LEFTY and CER1 work to create an A-P gradient of *Nodal* and *Wnt* signals in the epiblast. By blocking *Nodal* and *Wnt* signals, these antagonists promote neuroectodermal fates in the underlying epiblast. The *Wnt* and *Nodal* signals on the future posterior side of the embryo instruct cells to acquire mesodermal and endodermal fates.

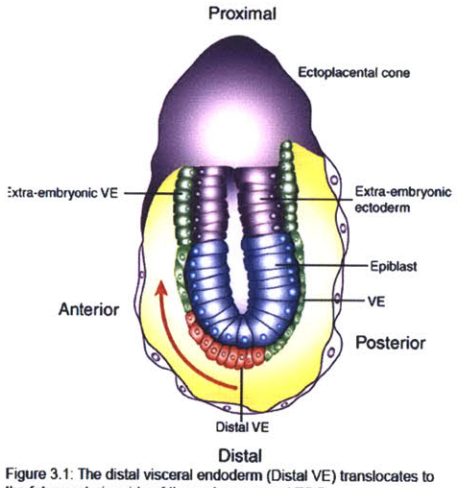


Figure 3.1: The distal visceral endoderm (Distal VE) translocates to the future anterior side of the embryo around E5.5.

If the distal visceral endoderm does not translocate properly , the A-P axis will not be established in the correct place because now the gradients of *Wnt* and *Nodal* will be aligned more closely with the proximal-distal (P-D) axis (Kinder et al., 2001). Growth of the embryo, instead of being directed along the A-P axis, would now be directed more along the P-D axis. The growth and cell movements occurring during gastrulation could result in the extrusion of

the prospective anterior region of the embryo from the surrounding extraembryonic endoderm as the body axis elongates. Thus, defective *Nodal-Lefty* signaling could result in out-of-the-yolk-sac embryos.

The action of AAGUGC seed miRNAs on *Nodal-Lefty* signaling is evolutionarily conserved from zebrafish to humans. In zebrafish, miR-430, targets both nodal agonist *squint* and its antagonist *lefty* to modulate nodal signaling during early development (Choi et al., 2007). In human ES cells depletion of *miR-302*, which targets *lefty1* and *lefty2*, leads to a strong decrease in the expression of mesodermal and endodermal markers (Barroso-delJesus et al., 2011).

Lefty1 and *Lefty2* are also known targets of the AAGUGC miR-290 miRNAs (Sinkkonen et al., 2008; Leung et al., 2011). Recent data from Ago2 Clip-seq experiments in mouse ES cells has experimentally validated not only *Lefty1* and *Lefty2* but also other inhibitors of the *TGFβ/Nodal* signaling pathway as miR-290 family targets (Leung et al., 2011). Based on ChIP-seq data from mouse ES cells, Marson and colleagues predicted that miR-290 miRNAs function in an incoherent feed-forward loop with *Oct4* and *Lefty1* (Marson et al., 2008). In this loop *Oct4* activates expression of both the *mir-290* cluster and *Lefty1,* and AAGUGC miR-290 miRNAs work to repress *Lefty1* expression.

The out-of-the-yolk-sac phenotype conferred by *mir-290-295* loss is consistent with the theory that miR-290 miRNAs might play a role in *Nodal-Lefty* signaling in the embryo. miR-290 miRNAs are expressed in both the extraembryonic (visceral) endoderm and epiblast around gastrulation (Spruce et al., 2010). Examining the expression of DVE markers would help to determine whether the DVE correctly translocates in *miR-290-295-/-* embryos. *Mir-290-295-/-* embryos could also be tested for increased levels of LEFTY1.

Lefty1, along with *Lefty2*, also work to set up the left-right (L-R) axis, the last of the three body axes to be established (Meno et al., 1998). *Mir-290-295-/-* animals display no gross

defects in left right isomerism. Therefore if *mir-290-295* is involved in *Lefty* regulation in the early embryo why do we not see any left-right isomerization defects in our homozygous mutants? Future experiments will need to address this question. L-R axis formation occurs at E8.0 (Takaoka et al., 2007), about 3 days later than A-P axis formation, so it is possible that the hypothesized *miR-290-295* mediated regulation of *Lefty1* does not occur at this time. (The exact spatial and temporal expression patterns of miR-290 miRNAs in the early embryo is currently not known.) It is also possible that the most severely affected *mir-290-295-/-* embryos die or arrest before the L-R axis is determined.

Potential causes of germ-cell mislocalization conferred by mir-290-295 deficiency

Mir-290-295 deficiency results in female specific sterility. Although *mir-290-295* homozygous male knockouts were fertile, the original germ-cell defect occurred during early germ-cell development in both sexes. Both male and female *mir-290-295-/-* showed reduced germ cells colonizing the gonads. Observations of migrating PGCs revealed a reduction in the number of correctly localized germ cells in *mir-290-295*-deficient mutants. In E9.5 male and female homozygous mutants, the decreased number of PGCs properly localized to the hindgut area correlated with the increased number of ectopic germ cells observed on the ventral posterior surface of the embryo. Correctly localized *mir-290-295-/-* PGCs behaved identically to wild-type PGCs in all aspects tested. Just like their wild-type counterparts, *mir-290-295-/-* PGCs correctly localized to the hindgut migrate in a posterior-to-anterior and ventral-to-dorsal fashion, moving out of the hindgut, into the dorsal mesentery, then splitting into two branches and colonizing the genital ridge. Data from E9.5 embryos indicate that the

mir-290-295-/- PGCs were not undergoing apoptosis but rather were actively cycling during this migratory period. Taken together, these data suggest that germ-cell loss observed in the mutant is due to mislocalization of a subpopulation of primordial germ cells, which are subsequently unable to colonize the gonad and therefore cannot contribute to the germ-cell pool.

The mislocalization of the majority of *mir-290-295-/-* PGCs is the earliest germ-cell defect observed in the mutants. The important observation is not merely that *mir-290-295-/-* PGCs were mislocalized; wild-type embryos displayed ectopic PGCs as well. Instead, the crucial distinction between wild-type and *mir-290-295-/-* embryos is the number of mislocalized germ cells. Mutant embryos displayed at least four times as many ectopic PGCs on the hindlimb/tail area as did wild-type embryos and furthermore, this increase correlated with a drastic decrease in the number of PGCs correctly localized to the hindgut. Therefore, any attempt to explain the effects of *mir-290-295* deficiency on germ-cell development must begin with trying to understand what causes the early mislocalization of the majority of *mir-290-295-/-* germ cells.

Ectopic germ cells, observed at E9.5, were not found internally but rather on the surface of the embryo. One day earlier at E8.5, mutant PGCs had not yet started migrating and were stuck in a tight cluster at the base of the allantois. Taken together, these data suggest that ultimately mutant PGCs failed to be incorporated into the hindgut. (Because of the E8.5 data, it is unlikely that *mir-290-295-/-* PGCs were incorporated into the hindgut and left prematurely.) What might cause the PGCs to remain outside the hindgut?

Unfortunately, little is known about the factors controlling the early germ-cell migration into the endoderm and subsequent incorporation into the hindgut. Identifying the cause of the germ-cell mislocalization is further complicated by the fact that germ-cell defects are occurring at the same developmental time as the early embryonic delays and out-of-yolk-sac

abnormalities. It is not clear how the early embryological problems associated with *mir-290-295* deficiency impact germ-cell migration. As mentioned previously, mouse embryos with a deficient number of epiblast cells initially show a developmental delay but are eventually able to catch up to their wild-type counterparts. These embryos also display partial sterility due to early germ-cell loss and retarded migration of PGCs (Tam and Snow, 1981).

To further understand the germ-cell mislocalization, it is necessary to explore whether this abnormality arises from cell-autonomous defects in the germ cells themselves or non-cell-autonomous defects. If the germ cells were completely non-motile or even slightly delayed in gaining motility, they would be unable to migrate into the endoderm in time to be incorporated into the hindgut. Given the location of the ectopic germ cells and the directions of tissue movement during this time (Downs and Harmann, 1997; Anderson et al., 2000), it seems unlikely that the *mir-290-295-/-* PGCs would be completely non-migratory. PGCs unable to migrate would most likely remain at the base of the allantois. Morphogenetic movement at the allanatois occurs in a proximal-to-distal fashion (Downs and Harmann, 1997). Therefore, non-motile PGCs would likely be pushed into the allantois rather than the hindlimb area where *mir-290-295-/-* ectopic germ cells are located.

Defective migratory cues in the surrounding environment could also affect the ability of the germ cells to move to the endoderm. Interestingly, miR-430, which possesses the same seed sequence as many of the miR-290 family miRNAs, has been implicated in control of zebrafish germ-cell migration (Staton et al., 2011). MiR-430 appears to regulate the expression of chemokines in the surrounding soma through which the germ cells migrate. It is possible that miR-290 miRNAs act in a similar manner.

It is also possible that *mir-290-295-/-* PGCs could be capable of migrating and have the correct cues but be physically blocked from entering the hindgut due to defective movement of hindgut endoderm. The *Sox17*-null mutant illustrates this point (Hara et al., 2009). *Sox17*

is a transcription factor critical for hindgut expansion. In *Sox17*-null embryos, about 10 PGCs at the vanguard are blocked from entering the hindgut by definitive endoderm cells that are incapable of moving from the inner mesoderm to the outer endoderm. As a result, the remainder of the PGCs cannot enter the hindgut and instead migrate around this blocked entrance into the yolk sac. This defect is rescued in chimeras of *Sox17*-null and wild-type cells, indicating that defective hindgut expansion can affect germ-cell migration. Any potential defect in the hindgut endoderm would need to be subtle, because no overt abnormalities of the hindgut were observed in *mir-290-295-/-* mice.

Unfortunately, the *mir-290-295* expression data cannot definitively distinguish between cell-autonomous and non-cell-autonomous defects. While *mir-290-295* has been shown to be expressed in the epiblast, it is not known whether or not *mir-290-295* is still expressed in the epiblast, near base of the allantois around late E7.5. Although *mir-290-295* has been shown to be expressed in migrating germ cells (Hanina et al., 2010), its expression in PGCs has not been verified prior to E9.5. Therefore, it is not known if the nascent E7.5-8.5 PGCs express *mir-290-295*. Even if nascent PGCs do express the miR-290 cluster miRNAs, the presence of DND-1 (Dead end 1) in these cells might prevent the miRNAs from exerting any regulatory effects. DND-1 is an RNA-binding protein that binds to the 3'UTR of mRNAs thereby preventing their association with miRNAs. *Dnd-1* has been shown to block the effects of miR-372, the miR-290 family ortholog, on LATS2 in a human germ-cell tumor line (Kedde et al., 2007). In addition, *Dnd-1* also prevents miR-430 from negatively regulating germ-cell-essential genes, *nanos1* and *tdrd7*, in the PGCs of zebrafish. In the mouse, *Dnd-1* is expressed in germ cells beginning at E6.75 (Yabuta et al., 2006) and *Dnd-1-/-* mice show a decrease in PGCs starting around E8.0 (Youngren et al., 2005).

Thus, any future work on the germ-cell phenotype conferred by *mir-290-295* deficiency must consider both the germ cells and the surrounding soma as possible contributors to the

PGC mislocalization. Generation of a conditional knockout mouse in which *mir-290-295* is deleted only in the germline would clearly determine whether loss of *mir-290-295* in the germ cells themselves or in the soma causes the mislocalization phenotype.

It is important to note that our experiments to date have not ruled out the possibility that the mismigration defect is an indirect effect of improper germ-cell specification. We do not know whether *mir-290-295-/-* embryos and wild-type embryos have the same number of PGCs prior to the start of migration. We also do not know whether early *mir-290-295-/-* PGCs express appropriate markers such as *Blimp1*, *Prdm14*, and other germ-cell-specification genes. Nor do we know whether or not early *mir-290-295-/-* PGCs accurately repress the somatic program or undergo the appropriate genetic modifications. Although *mir-290-295-/-* embryos display far greater amounts of AP-positive early PGCs than the *Blimp1* or *Prdm14* mutants (Ohinata et al., 2005; Vincent et al., 2005; Yamaji et al., 2008), we cannot rule out a role for *mir-290-295* in germ-cell specification based solely on this fact.

Partially Penetrant Phenotypes and Lack of Robustness

The phenotypes associated with *mir-290-295* deficiency can be characterized by incomplete penetrance and variable expressivity. About 75% of *mir-290-295-/-* embryos die before birth, but about 25% are able to withstand loss of *mir-290-295* and survive to adulthood. Some of the *mir-290-295* homozygous knockouts are severely delayed compared to their wild-type counterparts while other *mir-290-295* homozygous knockouts are only slightly delayed. Some *mir-290-295-/-* PGCs are mislocalized while others correctly migrate, colonize the gonad, and contribute to the gene pool.

This phenotypic diversity conferred by miRNA loss is consistent with the theory that miRNAs promote robustness. Robustness is defined as the ability of developmental processes to yield reproducible outcomes despite perturbations to the system (Shomron,

2010). These perturbations need not only be of external, environmental origin. Perturbations can also arise from intrinsic factors within the cell such as the inherently noisy processes of transcription and translation (Raj and van Oudenaarden, 2008). MiRNAs are ideal candidates for enhancing the stability of developmental systems for several reasons . (Leung and Sharp, 2007; Li et al., 2009; Leung and Sharp, 2010). First, miRNAs can generate a rapid response to fluctuations in the system. This is because microRNAs control the stability and translation of mRNAs that are often already present in the cell. Furthermore, the biogenesis of miRNAs is faster than that of proteins. Second, miRNAs are able to fine-tune the expression of their target genes. Third, one miRNA can regulate many targets, thereby allowing for a coordinated stress response.

MiRNAs are prevalent in feedback and feedforward motifs that have been implicated in stabilizing genetic regulatory networks (Tsang et al., 2007; Martinez et al., 2008). Furthermore, many miRNA loss-of-function mutants only demonstrate phenotypes under non-physiological, stress conditions (Leung and Sharp, 2007; Leung and Sharp, 2010). Zebrafish *miR-8* mutants appear wild-type under physiological conditions but fail to regulate osmotic stress (Flynt et al., 2009). Under high temperatures, Drosophila *miR-7* loss-of-function mutants show defects in eye development (Li et al., 2009). Loss of *miR-14* in flies causes sensitivity to salt imbalance (Xu et al., 2003). *miR-208* deficient mice cannot mediate hypertrophic growth in response to cardiac stress (van Rooij et al., 2007).

Not all of the experimental stresses were externally induced. Studies also demonstrated the requirement for miRNAs in buffering inherently stochastic physiological processes. *Drosophila miR-263a/b* protects against excessive apoptosis in the fly eye, thereby ensuring that sufficient numbers of eye-hair cells survive the pruning events that form the mature *Drosophila* eye (Hilgers et al., 2010). Zebrafish miR-430 was shown to promote robustness during germ-cell migration (Staton et al., 2011). In the zebrafish, the chemokine *Sdf1a*,

expressed in the soma, guides PGCs to the gonads. *Cxcr7* is expressed in the soma as well and is believed to function as a decoy receptor to bind Sdf1a and thereby refine the Sdf1a gradient. miR-430 represses both the ligand *Sdf1a* and its decoy receptor *Cxcr7.* Zebrafish lacking miR-430 have a reduced capacity to withstand alterations in the levels of Sdf1a or Cxcr7 and display mismigration of PGCs.

Considering the mislocalization of PGCs conferred by *mir-290-295* deficiency, it is possible that miR-290 miRNAs also play a role in regulating the robustness of germ-cell migration. SDF-1 in mice also acts as a cue to guide PGCs. However, *Sdf-1* function in mice is required for migration from the hindgut to the genital ridge, a time much later than when we observe germ-cell defects in *mir-290-295-/-* mice. Nevertheless, miR-290 miRNAs still might function like miR-430 to provide robustness to germ-cell migration. It is still not exactly clear why some mir-290-295-/- PGCs migrate correctly yet others do not. A role for miR-290 miRNAs in promoting robustness during germ-cell migration might help to explain this.

In 2010, Raj and colleagues provided the first experimental evidence that lack of robustness in gene-regulatory networks can confer partially penetrant phenotypes (Raj and van Oudenaarden, 2008). In the experiments of Raj and colleagues, loss of *skn-1,* a transcription factor which promotes intestinal fate in *C.elegans,* ultimately led to wide fluctuations in the expression of a downstream gene, *end1.* Expression of *end-1* was required to pass a certain threshold in order to produce an outcome, in this case, formation of intestinal cells. In some mutant embryos, *end-1* expression was high enough to promote intestinal cell formation while in other embryos *end-1* expression was too low to pass the threshold.

Since miRNAs have been shown to stabilize gene-regulatory networks and provide fidelity to developmental systems, it is possible that miR-290 miRNAs act in a buffering capacity *in vivo.* Loss of these miRNAs could lead to fluctuations in gene expression and this could

ultimately result in partially penetrant lethality. However, we do not have enough details about the origin of the embryonic lethality for the above statement to be more than a speculation. By pursuing some of the possible causes of embryonic lethality discussed earlier in this chapter we will be able to generate hypotheses to test whether or not miR-290 miRNAs act *in vivo* to promote robustness.

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Appendix

Reprogramming of Unipotent Spermatogonial Stem Cells to a Pluripotent State

Introduction

It is well known that primordial germ cells from E8.5-E12.5 embryos can be reprogrammed to pluripotent embryonic germ (EG) cells when removed from the embryo and cultured under appropriate conditions (Matsui et al., 1992; Resnick et al., 1992). It was originally thought that germ cells lose their ability to regain pluripotency around the time they enter the genital ridge because no EG cells had been successfully derived from germ cells older than E12.5. Several groups have now shown that it is also possible to obtain pluripotent cells from spermatogonial stem cells (SSCs) derived from neonatal and adult mouse testes (Kanatsu-Shinohara et al., 2004; Seandel et al., 2007; Ko et al., 2010) and from human testes (Conrad et al., 2008; Kossack et al., 2009). The first group to report this phenomenon, the Shinohara group, serendipitously discovered ES like cells growing in cultures of spermatogonial stem cells. These ES-like cells, termed multipotent germline stem (mGS), cells appeared to arise spontaneously at a very low frequency in their cultures. These mGS cells not only expressed ES-cell-specific markers but were also capable of contributing to the germline of chimeric mice.

Spermatogonial stem cells, from which mGS cells arise, are a unipotent cell type that give rise only to sperm (de Rooij and Russell, 2000). The mechanism by which these unipotent cells can regain pluripotency remains unknown. We originally chose to study this transition by culturing SSCs and attempting to find conditions to improve the efficiency of this conversion. However, in our hands this conversion was so rare as to occur only once in two years of culturing SSCs. During this time, the Yamanaka group reported the four-factor-mediated reprogramming of fibroblasts to a pluripotent state (Takahashi and Yamanaka, 2006). We then chose to determine whether or not the four Yamanaka factors, *Oct4*, *Sox2*, *Klf4,* and *c-Myc* could increase the efficiency of SSC to mGS transition.

Materials and Methods:

SSC Derivation and Cell Culture

Spermatogonial stem cell (SSC) lines were derived from the testes of neonatal mice according to an established protocol (Kanatsu-Shinohara et al., 2003). SSC lines could not be derived from mice that did not have a DBA or CD-1 strain background. SSC lines were grown in a special medium (Kanatsu-Shinohara et al., 2003) on irradiated mouse embryonic fibroblasts. SSC lines proliferated very slowly and were passaged with trypsin digestion approximately every four days.

Infections

SSCs were pre-plated on gelatin for 1-2 hours to remove the MEF feeder layer and then plated on laminin-coated plates for infection. SSCs were infected with doxycyline-inducible-FUW based lentiviruses encoding OCT4, SOX2, KLF4, and C-MYC (Brambrink et al., 2008). Two rounds of infection were performed lasting 10 hours each. Two days after the last infection, infected SSCs were transferred to MEF feeder layers and 24 hours later doxycyline (2µg/ml) was added to the cultures. Cells were cultured according to the Kanatsu-Shinohara protocol until ES-like colonies appeared. At this point the culture was switched to standard ES cell medium. Once colonies were large enough they were picked and expanded for analysis. Doxycycline was removed from the cultures around 15-20 days after initial infection.

Results and Discussion

Derivation of *in vitro* Spermatogonial Stem cell Cultures

Spermatogonial stem cell (SSC) cultures were derived from the testes of neonatal mice (in a DBA background) according to an established protocol (Fig. A1.A) (Kanatsu-Shinohara et al., 2003). These cell lines expressed cell-surface markers previously shown to be enriched in spermatogonial stem cell populations such as α6 integrin, β1 integrin, and EpCAM, in a manner comparable to established lines (Fig. A1.B) (Kanatsu-Shinohara et al., 2003). Furthermore, both our derived SSC line (LM) and an established SSC line (KSh) express *Plzf*, a transcription factor essential for SSC maintenance (Fig. A1.C).

According to Kanatsu-Shinohara and colleagues, SSCs are able to randomly and spontaneously convert to multipotent germline stem (mGS) cells. The rate of conversion is hard to estimate although Kanatsu-Shinohara and colleagues have estimated the frequency at 1 in 1.5x107 cells (equivalent to about 35 newborn testes) (Kanatsu-Shinohara et al., 2004). The original goal of our study was to examine the mechanisms underlying the SSC to mGS conversion. However, over the two years that we continually maintained SSC cultures only once was a conversion to the mGS state observed.

During the course of our experiments Yamanaka and colleagues published their groundbreaking study demonstrating that four transcription factors, OCT4, SOX2, C-MYC, and KLF-4 could reprogram murine fibroblasts to a pluripotent state (Takahashi and Yamanaka, 2006). Based on this exciting finding we set out to determine whether these four factors could force the transition from the unipotent SSC state to the pluripotent mGS state with high efficiency. Because SSCs had been reported to convert spontaneously, perhaps only one or two factors would be required to efficiently generate pluripotent stem cells. Before adding the four reprogramming factors we first sought to determine the endogenous levels of OCT4, SOX2, C-MYC, and KLF4 in our SSC lines.

SSCs express low levels of C-MYC and KLF-4 and negligible levels of OCT4 and SOX2

Immunoblots of SSC protein samples suggested that SSCs express C-MYC and KLF4, albeit at a lower level than that seen in the pluripotent ES and mGS cell lines (Fig. A.2 C-D). Both our SSC line (LM) and the established Kanatsu-Shinohara SSC line (KSh) express comparable amounts of C-MYC although the KSh line expresses slightly more KLF4 than our LM line. The discrepancy between the two lines was more noticeable when the levels of OCT4 and SOX2 were examined. The KSh line showed faint expression of OCT4 and SOX2 while our LM line did not show expression of either of these proteins. (Fig. A.2 A-B) This

could potentially explain why our LM line did not covert to the mGS state with higher frequency because even though the conversion rate reported by Kanatsu-Shinohara is quite low, we would have still expected to observe more than one conversion in two years. Interestingly, in our hands we never observed conversion of the purchased KSh line to an mGS state. Therefore perhaps there is some aspect of our culturing system which makes SSC lines in our hands less amenable to spontaneous reprogramming.

OCT4, SOX2, C-MYC, and KLF4 reprogram SSCs to a pluripotent state

In order to have temporal control over the expression of the exogenous reprogramming factors we chose to use the doxycyline-inducible M2rtTA system. SSC lines were derived from DBA background mice that expressed the M2rtTa transactivator from the Rosa26 locus (Beard et al., 2006). In the presence of doxycyline, the M2rtTA activator will bind to and activate transcription of loci containing tet operator sequences.

SSCs were infected with tetO FUW lentiviruses encoding the four factors (see scheme in Fig. A3.A). In these experiments, each factor was carried by a separate virus. After approximately 10 days of doxycyline induction, small roundish cells began to appear and by day 12 formed compact colonies with an ES-like morphology (Fig. A.3 B). No ES-like colonies appeared in infected cells not treated with doxycycline. Doxycyline was removed from the cultures around day 15-20 and most colonies maintained their ES like morphology. These (doxycyline-independent) cells expressed OCT4, SOX2, and NANOG (Fig. A.3 C-E) and were termed iPSSCs (induced pluripotent spermatogonial stem cells). iPSSCs expressed levels of *Oct4*, *Sox2*, and *Nanog* similar to those in ES cells and mGS cells (Fig. A.4 A). iPSSCs also downregulated expression of *Plzf*, a transcription factor required for spermatogonial stem cell identity. Microarray analysis showed that iPSSCs had a profile more similar to that of other pluripotent cells (ES, mGS) than to the unipotent SSCs (Fig. A.4 B).

Injection of iPSSCs into the blastocysts of BALB/c revealed that iPSSCs were capable of contributing to a chimeric mouse (Fig. A.4 C). The extent of chimerism in the different tissues of the mouse was not examined. (SSCs in contrast would not be able to contribute to a chimera (Kanatsu-Shinohara et al., 2004).) Taken together, these data suggest that SSCs can be reprogrammed to iPSSCs by OCT4, SOX2, C-MYC, and KLF4.

iPSSCs have male specific imprinting pattern

The possibility that a contaminating MEF in the cultures gave rise to the iPSSCs is highly unlikely given the fact that SSCs were usually cultured for at least a month (in order to obtain enough cells for these experiments) prior to infection. However, one way to rule out this possibility was to test the imprinting patterns of the iPSSCs. Since iPSSCs are derived from male germ cells they should have an androgenetic imprinting pattern like SSCs. From the limited amount of bisulfite analysis done to date, it appears that iPSSCs were derived from germ cells and not from a contaminating MEF (Fig. A.5). iPSSCs, like SSCs, show no DNA methylation at paternally imprinted genes such as *Peg3* and *Snrpn*. In contrast, both SSCs and iPSSCs showed methylation at maternally imprinted genes. iPS cells, because of their somatic origin, do show mixed methylation patterns for both paternally imprinted (H19) and maternally imprinted genes (*Snrpn* and *Peg3*). Additional bisulfate analysis should be done to show definitively that the iPSSCs have male-specific imprint pattern.

Conclusions

In this study we have shown that SSCs cultured *in vitro* can be reprogrammed to a pluripotent state by Oct4, Sox2, C-MYC, and KLF4. Given that SSCs have been shown to spontaneously convert to multipotent germline stem cells, this conclusion is not surprising. A more interesting question would be whether or not only one of the four reprogramming

factors could convert the SSCs to a pluripotent state. Since established SSC lines express low levels of OCT4 and possibly SOX2, perhaps a slight increase in either one of these factors would be able to force a conversion to a stem-cell state.

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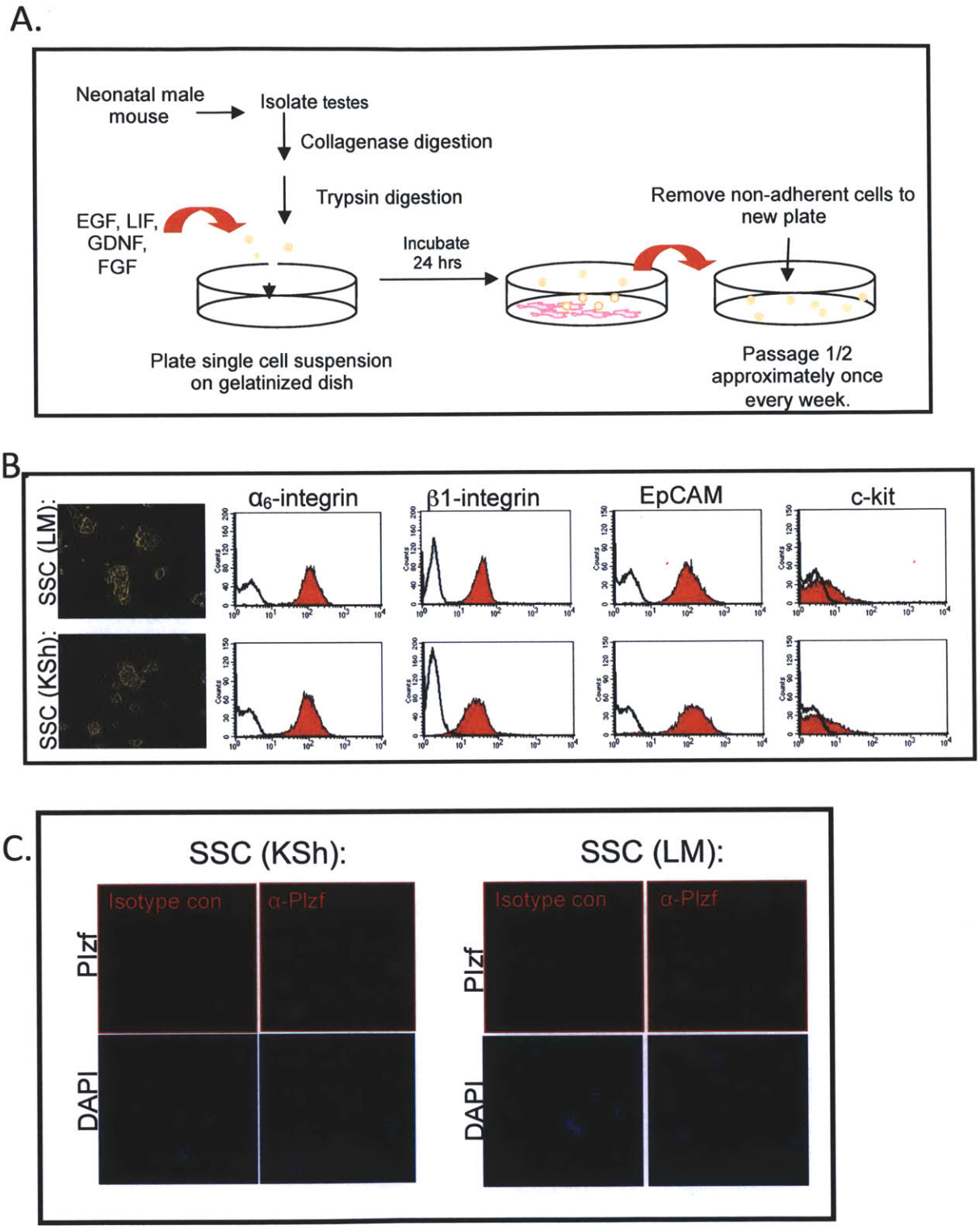

Figure A.1: (A) Procedure for deriving spermatogonial stem cell (SSC) lines. (B) Derived SSC lines (LM) express similar surface markers as established, purchased SSC lines (KSh). (C) Both derived SSC lines and established lines express *Plzf*, a transcription factor essential for SSC maintenance.

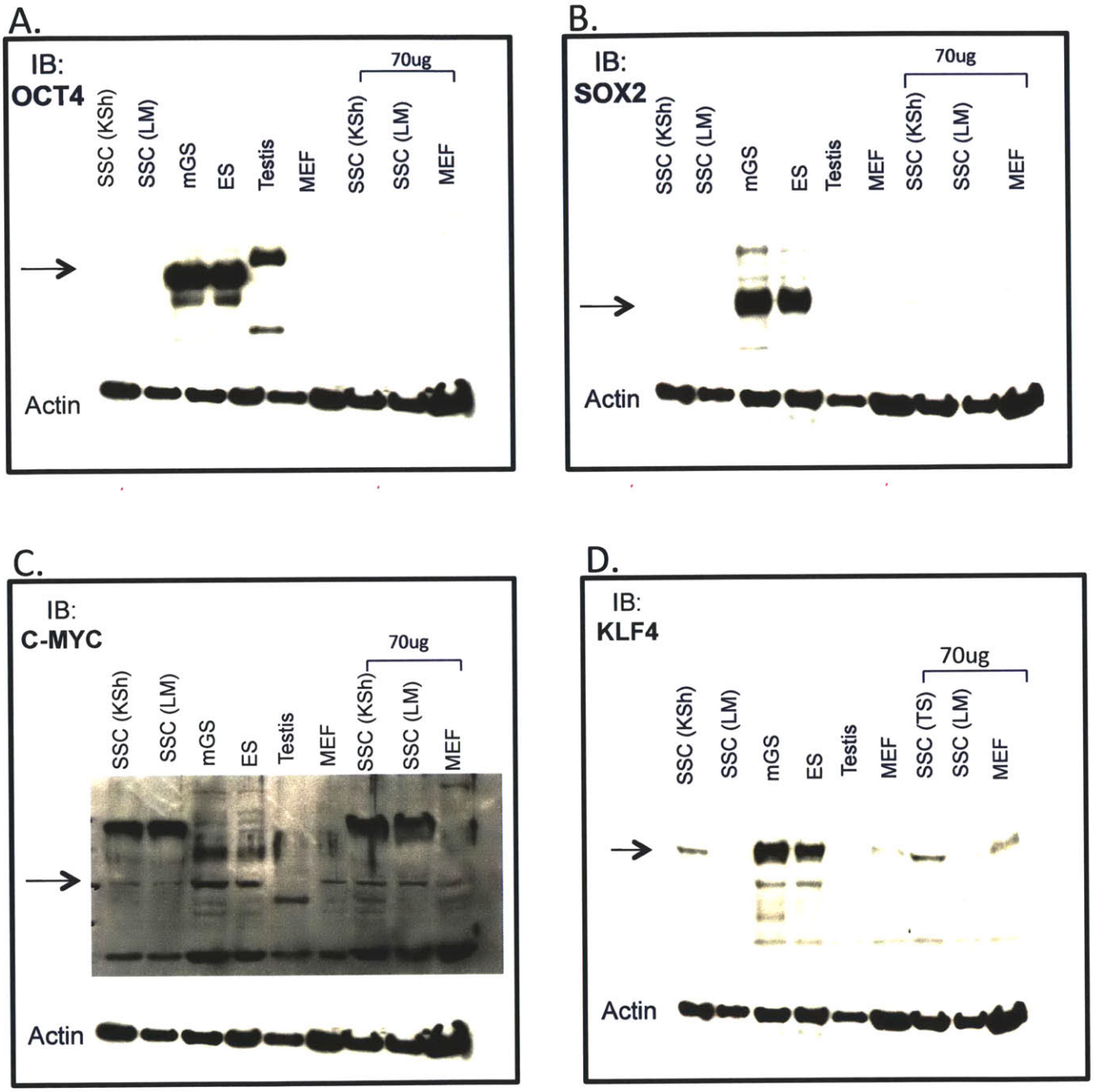

Figure A.2. SSC lines do not express appreciable levels of (A) OCT4, (B) SOX2, (C) C-MYC, or (D) KLF4 unlike their multipotent counterparts.

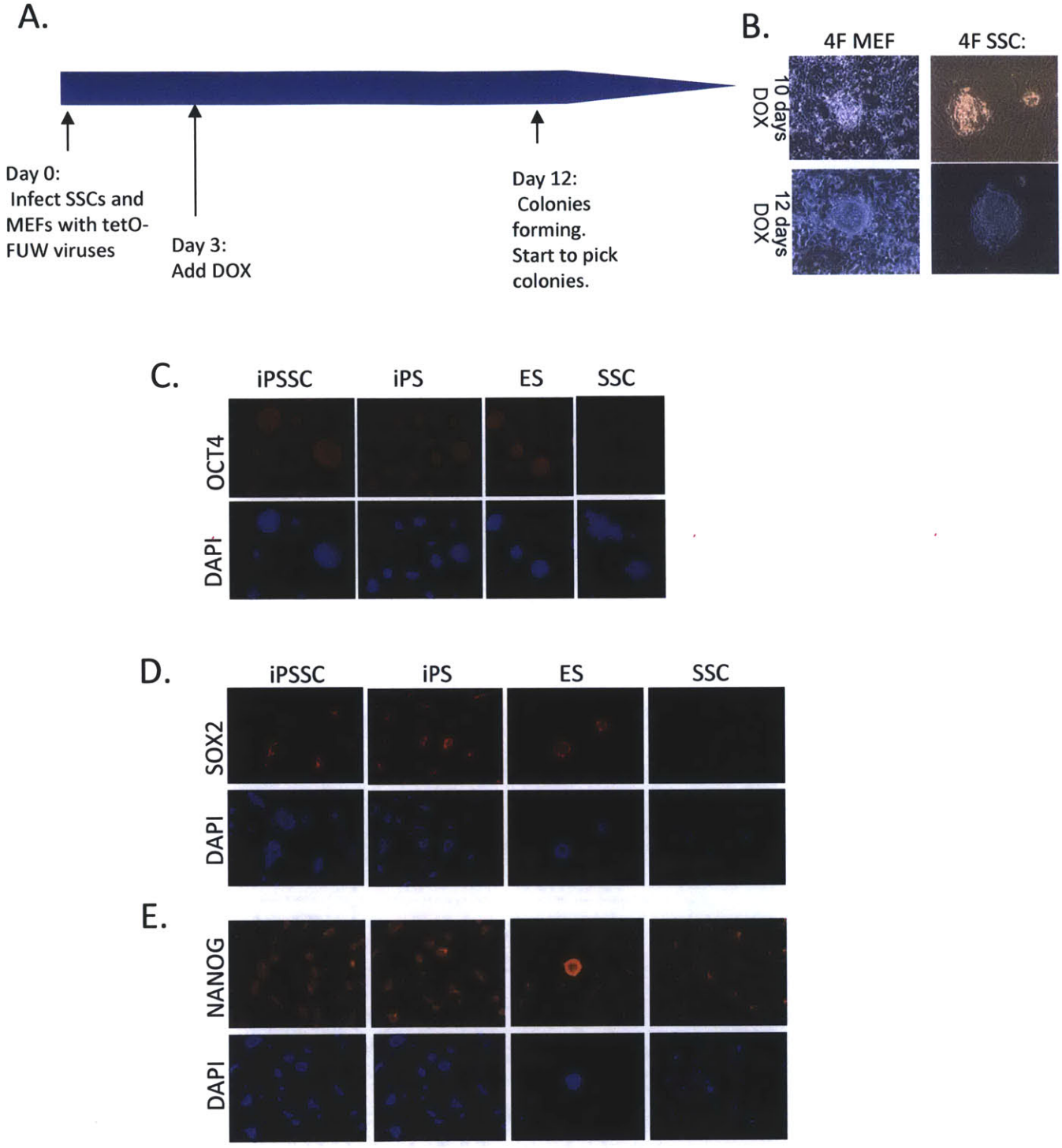

Figure A.3: Reprogramming of Spermatogonial Stem Cells. (A) Experimental outline for 4F infection experiments. (B) After 10 days doxycycline induction, ES –like colonies begin to form. Photographs of SSC and MEF cultures 10 and 12 days after doxycycline induction. (C-E) iPSSCs express OCT4 (C), SOX2 (D), and NANOG (E) like ES and iPS cells but unlike SSCs.

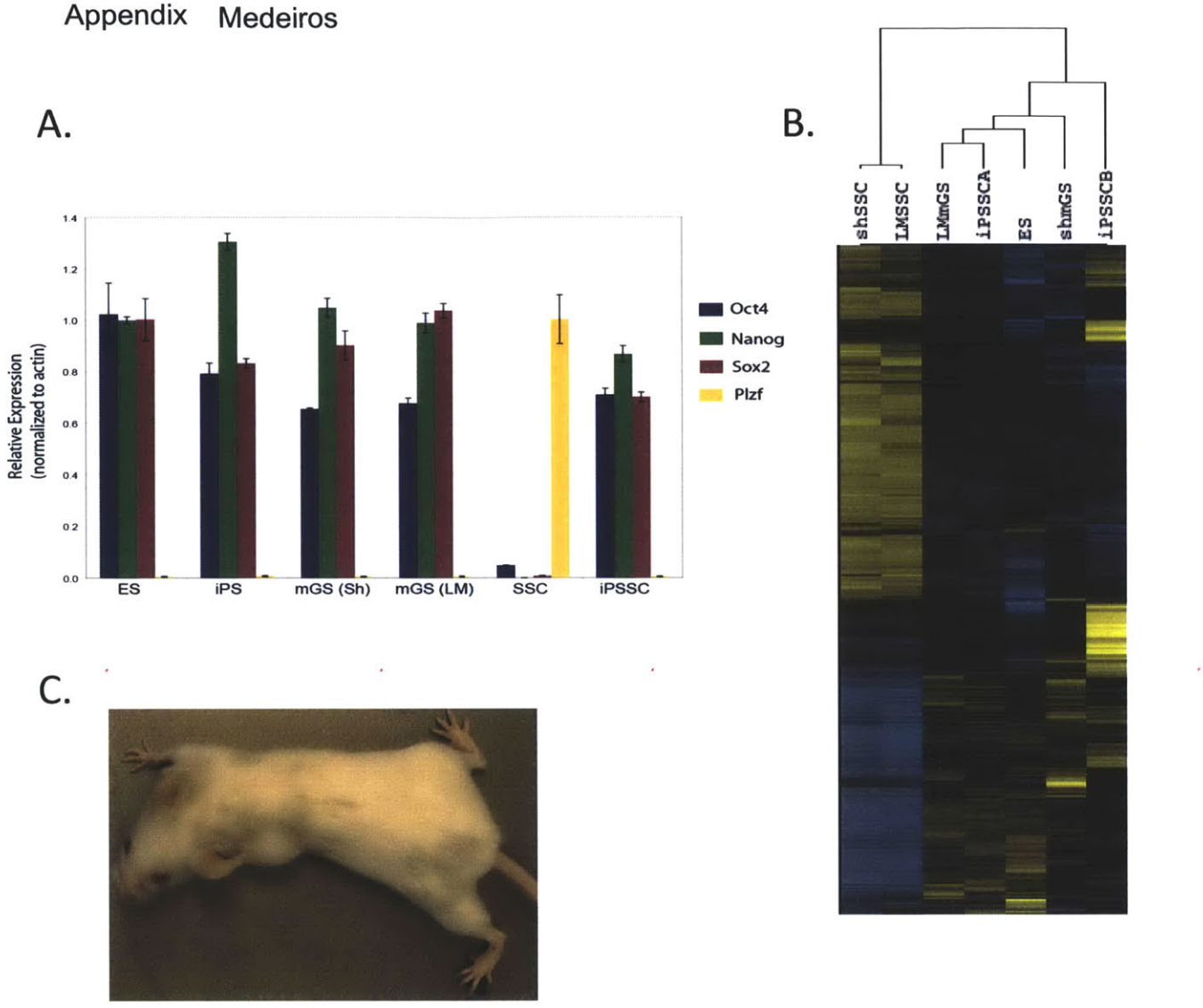

Figure A.4. iPSSCs are fully reprogrammed. (A) iPSSCs downregulate the SSC marker *Plzf* and upregulate pluripotency associated genes *Oct4*, *Sox2*, and *Nanog*. (B) iPSSCs have an expression profile more similar to pluripotent mGS and ES cells than unipotent SSCs. Heat map courtesy of Albert Cheng. (C) iPSSCs can contribute to mouse development. Note the brown spots of fur on the otherwise albino mouse.

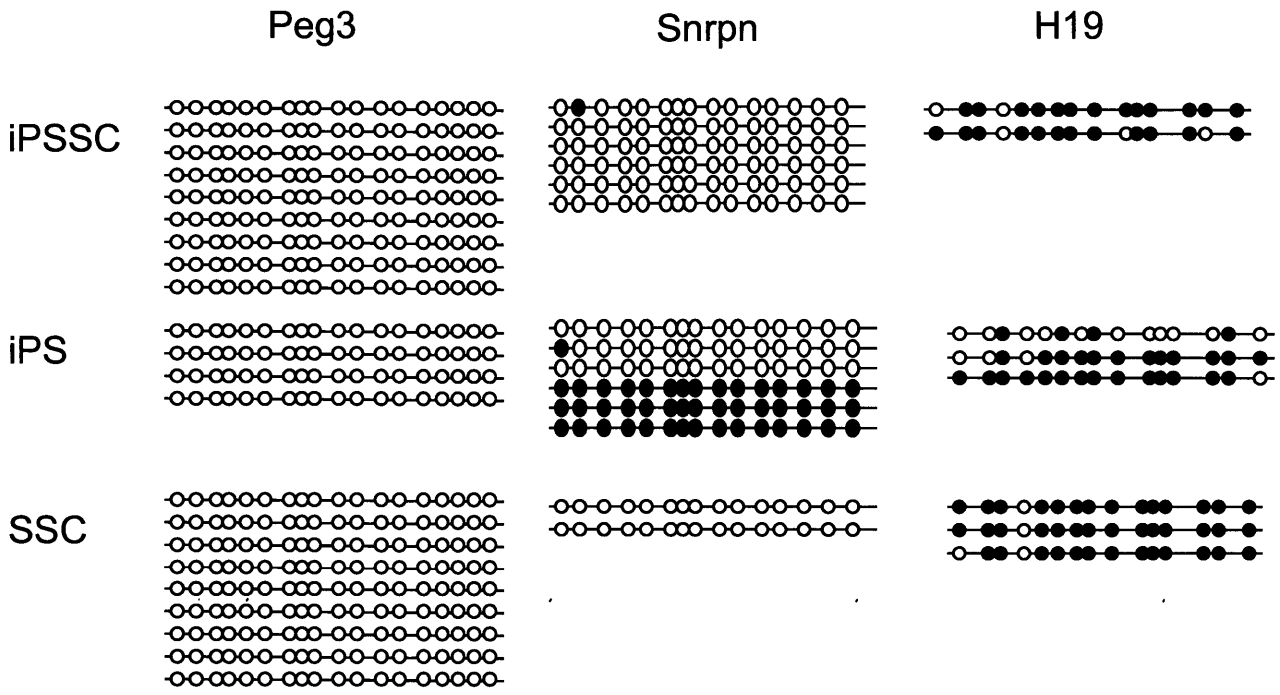

Figure A.5. Imprinting Patterns of iPSSCs are more similar to SSCs than iPSCs suggesting that iPSSCs were derived from SSCs and not contaminating fibroblasts. Peg 3 and Snrpn are maternally imprinted genes while H19 is paternally imprinted. Black circles indicate methylation.