1 Oocyte Development before and during Folliculogenesis

Melissa Pepling

1.1 Introduction

This chapter will focus on female germ cell development from the time the cells arrive at and populate the gonad through primordial follicle formation and initial activation as well as cyclical activation. The same basic events occur in most species but with variation in the timing as shown in Table 1.1. The migration of the primordial germ cells (PGCs) to the gonad will not be discussed here. Sex determination will also not be covered. A great deal of work on female germ cell development has been performed in rodents; thus the state of knowledge in rodents will be discussed first followed by information from domestic species.

1.2 Germ Cell Cyst and Ovigerous Cord Formation

In the mouse, PGCs arrive at the genital ridge starting at 10.5 days post coitum (dpc) and divide by mitosis until 13.5 dpc (Figure 1.1a) (Monk & McLaren, 1981). During this time germ cells are classified as oogonia and develop as clusters of interconnected cells called germ cell cysts (Figures 1.1b and 1.3a) (Pepling & Spradling, 1998). Germ cell cysts have been well studied in male and female invertebrates (de Cuevas et al., 1997). In the Drosophila female, cysts are formed from germline stem cells that divide to produce a daughter stem cell and a cyst forming cell called a cystoblast. The cystoblast undergoes four synchronous mitotic cell cycles. However, after each division, cytokinesis is incomplete so that the cells remain connected by intercellular bridges. Only one cell of the cyst will become an oocyte, the remaining cells serve as nurse cells, supplying nutrients to the oocyte through the intercellular bridges.

Mouse female germ cells share several characteristics of the Drosophila germline cysts, including synchronous divisions, incomplete cytokinesis, intercellular bridge connections, and transport of molecules and organelles across bridges (Pepling & Spradling, 1998). Unlike Drosophila, the number of cells per cyst seems to be variable, and synchrony is lost in some dividing cyst cells. It is also unclear if some of the germ cells of the cyst serve as nurse cells in the mouse. In Drosophila, the future oocyte increases in size as it receives cytoplasm from the nurse cells (de Cuevas et al., 1997) but this does not appear to happen in the mouse (Pepling & Spradling, 2001). As the oogonia divide and form germ cell cysts, the cell clusters become enclosed in ovigerous cords consisting of epithelial pregranulosa cells surrounded by a basal lamina (Mazaud et al., 2005). There are three
Table 1.1  Timing of female germ cell development in humans, mice, and several domestic species (days of gestation).

<table>
<thead>
<tr>
<th></th>
<th>Cow</th>
<th>Sheep</th>
<th>Pig</th>
<th>Goat</th>
<th>Horse</th>
<th>Mouse</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arrial at gonad</td>
<td>35 (Erickson, 1966)</td>
<td>23 (Juengel et al., 2002)</td>
<td>18 (Black &amp; Erickson, 1968)</td>
<td>25 (Lee et al., 1998)</td>
<td>22 (Curran et al., 1997)</td>
<td>10.5 (Monk &amp; McLaren, 1981)</td>
<td>28 (Witschi, 1948)</td>
</tr>
<tr>
<td>Germ cell cysts /ovigerous cords</td>
<td>57–90 (Garverick et al., 2010; Russe, 1983)</td>
<td>38–75 (Juengel et al., 2002; Sawyer et al., 2002)</td>
<td>20–50 (Black &amp; Erickson, 1968)</td>
<td>35–90 (Pailhoux et al., 2002)</td>
<td>*</td>
<td>10.5–13.5 (Mazaud et al., 2005; Pepling &amp; Spradling, 1998)</td>
<td>50–140 (Hartshorne et al., 2009)</td>
</tr>
<tr>
<td>Meiotic entry</td>
<td>75–82 (Erickson, 1966; Russe, 1983)</td>
<td>55 (Sawyer et al., 2002)</td>
<td>47 (Bielanska-Osachowska, 2006)</td>
<td>55 (Pailhoux et al., 2002; Pannetier et al., 2006)</td>
<td>60 (Deanesly, 1977)</td>
<td>13.5 (McLaren, 2000)</td>
<td>70 (Hartshorne et al., 2009)</td>
</tr>
<tr>
<td>Follicle formation</td>
<td>90 (Yang &amp; Fortune, 2008)</td>
<td>66–75 (Juengel et al., 2002; Russe, 1983)</td>
<td>56–68 (Bielanska-Osachowska, 2006; Oxender et al., 1979)</td>
<td>90 (Pailhoux et al., 2002; Pannetier et al., 2006)</td>
<td>102 (Deanesly, 1977)</td>
<td>17.5 (Pepling et al., 2010)</td>
<td>140 (Gillman, 1948; Gondos et al., 1971; Witschi, 1963)</td>
</tr>
<tr>
<td>Follicle development (first wave)</td>
<td>140 (Yang &amp; Fortune, 2008)</td>
<td>100 (Sawyer et al., 2002)</td>
<td>75–90 (Ding et al., 2010; Oxender et al., 1979)</td>
<td>*</td>
<td>*</td>
<td>17.5 (Pepling et al., 2010)</td>
<td>150 (Hartshorne et al., 2009)</td>
</tr>
<tr>
<td>Gestation</td>
<td>280</td>
<td>145</td>
<td>112</td>
<td>150</td>
<td>340</td>
<td>19.5</td>
<td>280</td>
</tr>
</tbody>
</table>

* indicates unknown.
Primordial germ cells arrive at genital ridge.

(a) Primordial germ cells arrive at genital ridge.

(b) Germ cell cysts and ovigerous cords form. Germ cells enter meiosis.

(c) Germ cell cysts break apart and follicles form.

Possible sources of pregranulosa cells in the mouse: the rete ovarii that connects to the mesonephrous, mesenchymal cells of the gonad, or ovarian surface epithelium (Liu et al., 2010). The pregranulosa cells may come from one or all three of these sources and may vary depending on the species (Sawyer et al., 2002).

The molecular control of ovigerous cord and germ cell cyst formation is not well understood. As the PGCs arrive at the gonad, several signaling pathways control their numbers such as Kit signaling, Fibroblast growth factor (FGF) signaling, and the interleukin pathway (see Table 1.2) (Farini et al., 2005; Merkwitz et al., 2011; Takeuchi et al., 2005). In addition, both Oct4 and Nanos3 block germ cell survival.

**Table 1.2 Genes involved germ cell survival.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein/ Function</th>
<th>Female Mutant Phenotype</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>bcl-x</td>
<td>Anti-apoptotic B-cell leukemia/lymphoma 2 (Bcl2) family member.</td>
<td>Germ cell loss by 15.5 dpc.</td>
<td>(Rucker et al., 2000)</td>
</tr>
<tr>
<td>β-catenin</td>
<td>Wnt signaling pathway.</td>
<td>Germ cell loss starting at 16.5 dpc, sex reversal.</td>
<td>(Liu et al., 2009)</td>
</tr>
<tr>
<td>fgf2r-IIIb</td>
<td>FGF signaling pathway.</td>
<td>Reduced number of germ cells at 11.5 dpc.</td>
<td>(Takeuchi et al., 2005)</td>
</tr>
<tr>
<td>follistatin</td>
<td>Activin antagonist, TGFβ family member.</td>
<td>Germ cell loss starting at 16.5 dpc, sex reversal.</td>
<td>(Yao et al., 2004)</td>
</tr>
<tr>
<td>kit</td>
<td>Kit oncogene, receptor tyrosine kinase.</td>
<td>Reduced number of germ cells</td>
<td>(Merkwitz et al., 2011)</td>
</tr>
<tr>
<td>kitl</td>
<td>Kit ligand, Stem cell factor.</td>
<td>Reduced number of germ cells</td>
<td>(Merkwitz et al., 2011)</td>
</tr>
<tr>
<td>nanos3</td>
<td>Nanos family of RNA binding proteins</td>
<td>Reduced number of germ cells at 10.5 dpc.</td>
<td>(Suzuki et al., 2008)</td>
</tr>
<tr>
<td>oct4</td>
<td>Pou domain transcription factor</td>
<td>Reduced number of germ cells at 10.5 dpc.</td>
<td>(Kehler et al., 2004)</td>
</tr>
<tr>
<td>rspo1</td>
<td>R-spondin homolog 1.</td>
<td>Germ cells do not enter meiosis, sex reversal.</td>
<td>(Chassot et al., 2008)</td>
</tr>
<tr>
<td>wnt4</td>
<td>Wnt, secreted glycoprotein family, wnt signaling pathway.</td>
<td>Germ cell loss starting at 16.5 dpc, sex reversal.</td>
<td>(Tomizuka et al., 2008)</td>
</tr>
</tbody>
</table>
cells from undergoing apoptosis (Kehler et al., 2004; Suzuki et al., 2008) whereas TGFβ1 and activin prevent proliferation of PGCs in culture (Richards et al., 1999). Several genes have also been implicated in the control of germ cell survival later, after arrival at the ovary (Table 1.2). Two B-cell leukemia/lymphoma 2 (Bcl2) family members, Bcl-x and Bax, have been implicated in the regulation of germ cell survival (Rucker et al., 2000). Bcl-x hypomorphs lose their germ cells by 15.5 dpc but when mice lack both bcl-x and bax, germ cell numbers are restored. Other cell death regulators such as Bcl2 and Caspase 2 have been implicated in oocyte survival in the adult ovary (Bergeron et al., 1998; Ratts et al., 1995). There are also several genes that affect germ cell survival in the ovary slightly later, with loss starting at 16.5 dpc in mouse mutants of β-catenin, follistatin, r-spondin homolog 1 (rspo1), and wnt4 (Chassot et al., 2008; Liu et al., 2009; Tomizuka et al., 2008; Yao et al., 2004). In addition, testes like characteristics are observed in mutants lacking these genes.

In cattle, germ cells begin to arrive at the gonad at approximately 35 days of gestation (Erickson, 1966). From arrival at the gonad to follicle formation, germ cell numbers increase from 16,000 to 2,700,000. Like mice, the bovine germ cells exhibit some of the key characteristics of germ cell cysts such as synchronous divisions and intercellular bridge connections (Russe, 1983). The germ cell clusters are observed starting at approximately 57 to 60 days of gestation (Garverick et al., 2010; Russe, 1983). The developing oogonia are surrounded by epithelial cells to form ovigerous cords at approximately 60 days of development (Garverick et al., 2010).

In a similar manner, in sheep, PGCs arrive at the gonad at about 23 days of development (Juengel et al., 2002). Again, ovine germ cells develop in nests and appear similar in morphology to mouse germ cell cysts. The germ cells continue to divide and reach their maximum number of 805,000 at day 75 (Smith et al., 1993). Ovigerous cords form from 38 to 75 days (Sawyer et al., 2002). Somatic cells contact germ cells by desmasomes and the germ cell somatic cell complexes progressively fuse to form the ovigerous cords. The somatic cells surround the germ cells and secrete a basal lamina similar to other species. There has been some question as to the source of the somatic cells that form the ovigerous cords in sheep. Cells from the mesonephrous stream in to the developing ovary, and it was thought that these cells were the cells that surrounded the germ cells (Sawyer et al., 2002). However, ovarian surface epithelial cells are also thought to be a source of somatic cord cells in the sheep, and it is likely that both cell populations contribute.

Oogonia in pigs, goats, and horses have not been as well studied as other species but there are a few reports describing this stage of development. Porcine germ cells are observed in the region of the genital ridge as early as embryonic day 18 (Black & Erickson, 1968). Mitotic divisions begin at approximately 20 days, and germ cells increase in number from 5,000 cells to 1,100,000 by 50 days. As in other species, oogonia develop in clusters and by electron microscopy have been shown to be connected by intercellular bridges at 47 days (Bielanska-Osuchowska, 2006). In goats, oogonia clusters are observed in ovigerous cords between about 35 to 90 days (Pailhoux et al., 2002). Germ cells have also been observed to develop in clusters in the horse (Deanesly, 1977).

1.3 Meiotic Entry and Progression

In the mouse, oogonia stop dividing and begin to enter meiosis at 13.5 dpc and are then considered oocytes. The oocytes remain in germ cell cysts as they enter meiosis. Oocytes progress through the stages of prophase I of meiosis (leptotene, zygotene, and pachytene) and arrest in the diplotene stage. Oocytes begin to enter the diplotene stage at 17.5 dpc, and most have reached diplotene by PND5 (Borum, 1961). The oocytes remain arrested in the diplotene stage, and meiosis is not resumed until right before ovulation in response to a surge in luteinizing hormone (LH).
Table 1.3  Genes involved in meiotic entry and progression.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein/Function</th>
<th>Female Mutant Phenotype</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>atm</td>
<td>Ataxia-telangiectasia mutated homolog, involved in recombination and mismatch repair.</td>
<td>Sterile, germ cells arrest at pachytene and eventually die.</td>
<td>(Barlow et al., 1998)</td>
</tr>
<tr>
<td>cyp26b1</td>
<td>Cytochrome P450, family 26, subfamily B, degrades retinoic acid.</td>
<td>Perinatal lethal. Female germ cells prematurely express Stra8.</td>
<td>(Bowles et al., 2006; MacLean et al., 2001)</td>
</tr>
<tr>
<td>dmc1</td>
<td>Disrupted meiotic cDNA 1 (recA homolog), involved in recombination and mismatch repair.</td>
<td>Sterile, germ cells arrest at pachytene and eventually die.</td>
<td>(Pittman et al., 1998; Yoshida et al., 1998)</td>
</tr>
<tr>
<td>msh4</td>
<td>MutS homolog 4, involved in recombination and mismatch repair.</td>
<td>Sterile, germ cells arrest at pachytene and eventually die.</td>
<td>(Kneitz et al., 2000)</td>
</tr>
<tr>
<td>msh5</td>
<td>MutS homolog 5, involved in recombination and mismatch repair.</td>
<td>Sterile, germ cells arrest at pachytene and eventually die.</td>
<td>(de Vries et al., 1999)</td>
</tr>
<tr>
<td>stra8</td>
<td>Stimulated by Retinoic acid, gene 8.</td>
<td>Sterile, germ cells do not enter meiosis.</td>
<td>(Baltus et al., 2006; Menke et al., 2003)</td>
</tr>
<tr>
<td>sycp1</td>
<td>Synaptonemal complex protein 1.</td>
<td>Sterile, lack oocytes.</td>
<td>(de Vries et al., 2005)</td>
</tr>
<tr>
<td>sycp3</td>
<td>Synaptonemal complex protein 3.</td>
<td>Reduced fertility, defective meiotic chromosome segregation.</td>
<td>(Yuan et al., 2002)</td>
</tr>
</tbody>
</table>

The mechanisms controlling meiotic entry have started to be uncovered (Table 1.3). Entry into meiosis is thought to be regulated by retinoic acid, which induces female cells to begin meiosis (Bowles et al., 2006; Koubova et al., 2006). When the Retinoic Acid Receptor is blocked using an antagonist in ovary organ culture, female germ cells do not enter meiosis. Males express Cytochrome P450, family 26, subfamily B (Cyp26b1), which degrades retinoic acid, thereby preventing male germ cells from entering meiosis. In the ovary, meiotic entry occurs in a wave from anterior to posterior (Bullejos & Koopman, 2004; Menke et al., 2003). Retinoic acid upregulates a cytoplasmic protein called Stimulated by Retinoic Acid, gene 8 (Stra8) in females (Baltus et al., 2006). Stra8 is expressed first in the anterior moving posterior reflecting the wave of meiotic entry (Menke et al., 2003). Stra8 plays a role in premeiotic DNA replication and in chromosome cohesion and synapsis (Baltus et al., 2006).

Defects in female germ cell development are observed in mutants of several genes involved in DNA mismatch repair and recombination including ataxia-telangiectasia mutated homolog (atm), disrupted meiotic cDNA 1 (dmc1), mutS homolog 4 (msh4), and msh5 (Table 1.3) (Barlow et al., 1998; de Vries et al., 1999; Kneitz et al., 2000; Pittman et al., 1998; Yoshida et al., 1998). Males and females are sterile, and female germ cells arrest in the pachytene stage of meiotic prophase at 16.5 dpc. Eventually, the germ cells are lost in the mutants. During meiotic prophase, homologous chromosomes are held together by the synaptonemal complex. Two structural components of the synaptonemal complex, Synaptonemal complex protein (Scyp) 1 and Scyp3, are required for normal fertility. scyp1 mutants are sterile, and females lack oocytes (de Vries et al., 2005). In rats, inhibition of Scyp1 caused premature arrival at the diplotene stage and premature primordial follicle assembly, suggesting a link between cell cycle stage and primordial follicle development (Paredes et al., 2005). scyp3 mutants have reduced fertility, and although the oocytes appear to develop normally, chromosome segregation does not occur properly (Yuan et al., 2002).
In bovine ovaries, germ cells begin to enter meiosis starting at 75 to 82 days of gestation (Erickson, 1966; Russe, 1983). Entry into meiosis seems to be a gradual and prolonged process with some cells still found in mitosis even at birth. In sheep, meiotic germ cells are first observed at 55 days though mitotic germ cells are still observed up to 90 days (Juengel et al., 2002; Sawyer et al., 2002). The germ cells farthest from the surface epithelium are the first to enter meiosis. In porcine ovaries, germ cells begin to enter meiosis at 47 days of gestation (Bielsanska-Osuchowska, 2006), in the goat at 55 days (Pailhoux et al., 2002; Pannetier et al., 2006), and in the horse at 60 days of gestation (Deanesly, 1977).

1.4 Follicle Formation

The oocytes in germ cell cysts eventually separate, a process termed cyst breakdown, and become enclosed in primordial follicles consisting of one oocyte and several somatic granulosa cells (Figures 1.1c and 1.3b) (Pepling & Spradling, 2001). During this process, some cells in each cyst die by programmed cell death, with only a third of the total surviving. In one model, one cell of a cyst dies and breaks the large cyst into smaller cysts. This is repeated until a few individual oocytes remain. Thus, programmed cell death would be required for oocytes to break apart. Some cyst cells may support oocytes and eventually die, analogous to nurse cells in Drosophila. Programmed cell death during female germ cell development is common in many species including domestic species (Buszczak & Cooley, 2000). In mouse, cyst breakdown and oocyte loss occur concurrently, suggesting they are part of a regulated process. Mechanisms governing oocyte death remain uncharacterized. Work examining mutants lacking the programmed cell death regulator, Bax (a pro-death protein) suggests that oocyte cell death is required for cyst breakdown (Greenfeld et al., 2007). bax mutant ovaries have more oocytes than wild-type ovaries still in cysts, supporting the idea that programmed cell death is required for cyst breakdown.

In the mouse, oocyte loss and cyst breakdown begin in the medullary region of the ovary as early as 17.5 dpc (De Felici et al., 1999; Ghafari et al., 2007; McClellan et al., 2003; Pepling et al., 2010). In addition, follicles begin to form in the innermost region of the ovary at 17.5 dpc. The somatic pregranulosa cells surrounding the germ cells to form the ovigerous cords now begin to surround individual oocytes and become granulosa cells. In addition, before follicle formation, pregranulosa cells extend cytoplasmic protrusions between oocytes and may be involved in separating oocytes (Pepling & Spradling, 2001). There are regional differences in oocyte development, and oocytes located in the inner cortex and medullary regions of the ovary enter meiosis and start to grow first (Nandedkar et al., 2007; Peters, 1969). This regional pattern is set up between 13.5 and 16.5 dpc in the mouse concurrent with meiotic entry (Byskov et al., 1997). Oocytes in the resultant primordial follicles are thought to represent the entire pool available to a female during her reproductive life (Kezele et al., 2002).

Several mouse mutants have been generated that have ovaries with multiple oocyte follicles (MOFs) consisting of abnormal follicles with more than one oocyte (see Table 1.4). The oocytes in these follicles are believed to be remnants of germ cell cysts that did not completely break apart, resulting in more than one oocyte being enclosed in a follicle (Jefferson et al., 2006). This suggests that the genes disrupted in these mutants play a role in promoting cyst breakdown and primordial follicle formation. MOFs are observed in mutants of two members of the Transforming Growth Factor β (TGFβ) family, bone morphogenetic protein 15 (bmp15) and growth and differentiation factor 9 (gdf9) (Yan et al., 2001). Treatment of ovaries with another TGFβ family member, Activin A, promotes follicle formation (Bristol-Gould et al., 2006). In contrast, overexpression of
## Table 1.4 Genes involved in primordial follicle formation.

<table>
<thead>
<tr>
<th>Protein/Function</th>
<th>Female Mutant Phenotype</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ahr</em></td>
<td>Aryl Hydrocarbon Receptor, basic helix-loop-helix transcription factor. Reduced fertility, accelerated primordial follicle formation.</td>
<td>(Benedict et al., 2000; Robles et al., 2000)</td>
</tr>
<tr>
<td><em>akt</em></td>
<td>Serine/threonine kinase, also known as Protein Kinase B (PKB). Multiple oocyte follicles.</td>
<td>(Brown et al., 2010)</td>
</tr>
<tr>
<td><em>bax</em></td>
<td>Proapoptotic Bcl2 family member. Increased germ cell numbers, reduced follicle formation.</td>
<td>(Greenfeld et al., 2007)</td>
</tr>
<tr>
<td><em>bmp15</em></td>
<td>Bone Morphogenetic Protein 15, TGFβ family member. Multiple oocyte follicles.</td>
<td>(Yan et al., 2001)</td>
</tr>
<tr>
<td><em>dax</em></td>
<td>Dosage-sensitive sex reversal, adrenal hypoplasia critical region on chromosome X, gene 1, orphan steroid hormone receptor. Multiple oocyte follicles.</td>
<td>(Yu et al., 1998)</td>
</tr>
<tr>
<td><em>figla</em></td>
<td>Factor in the germ line alpha, folliculogenesis specific basic helix-loop-helix. Sterile, defective follicle formation, perinatal oocyte loss.</td>
<td>(Soyal et al., 2000)</td>
</tr>
<tr>
<td><em>follistatin</em></td>
<td>Activin antagonist, TGFβ family member. Reduced fertility, reduced follicle formation.</td>
<td>(Kimura et al., 2011)</td>
</tr>
<tr>
<td><em>foxl2</em></td>
<td>Forkhead box L2, winged helix transcription factor. Sterile, defective follicle formation, oocyte loss.</td>
<td>(Schmidt et al., 2004; Uda et al., 2004)</td>
</tr>
<tr>
<td><em>gdf9</em></td>
<td>Growth differentiation factor 9, TGFβ family member. Multiple oocyte follicles.</td>
<td>(Yan et al., 2001)</td>
</tr>
<tr>
<td><em>lunatic fringe</em></td>
<td>Regulator of Notch signaling. Multiple oocyte follicles.</td>
<td>(Hahn et al., 2005)</td>
</tr>
<tr>
<td><em>ngf</em></td>
<td>Nerve growth factor, neurotrophin signaling. Reduced follicle formation.</td>
<td>(Dissen et al., 2001)</td>
</tr>
<tr>
<td><em>nobox</em></td>
<td>Newborn ovary homeobox-encoding gene. Sterile, delayed follicle formation, oocyte loss.</td>
<td>(Rajkovic et al., 2004)</td>
</tr>
<tr>
<td><em>ntk1</em></td>
<td>NGF receptor, neurotrophin signaling. Reduced follicle formation.</td>
<td>(Kerr et al., 2009)</td>
</tr>
<tr>
<td><em>ntk2</em></td>
<td>Receptor for NT-4 and BDNF, neurotrophin signaling. Reduced follicle formation, reduced number of germ cells.</td>
<td>(Kerr et al., 2009; Spears et al., 2003)</td>
</tr>
<tr>
<td><em>p27</em></td>
<td>Cyclin-dependent kinase (Cdk) inhibitor 1, downstream of PI3K signaling. Progressive loss of fertility, accelerated primordial follicle formation.</td>
<td>(Rajareddy et al., 2007)</td>
</tr>
</tbody>
</table>

The Activin antagonist, Inhibin B, causes an increase in MOFs (McMullen et al., 2001). In addition, *follistatin* mutants are subfertile and have a delay in cyst breakdown and follicle formation (Kimura et al., 2011). Mutation of a regulator of Notch signaling, *lunatic fringe*, also results in the appearance of MOFs, suggesting that the Notch signaling pathway may be important in cyst breakdown and primordial follicle assembly (Hahn et al., 2005). Supporting this idea, inhibition of Notch signaling in culture caused a reduction in primordial follicle formation (Trombly et al., 2008). Thus, TGFβ and Notch signaling pathways play a role in cyst breakdown and primordial follicle formation.

There is also evidence for Neurotrophin signaling in the regulation of cyst breakdown and primordial follicle formation (Table 1.4). Mutation of the Neurotrophin, Nerve Growth Factor (NGF) resulted in females with fewer oocytes enclosed in primordial follicles and more oocytes still in germ cell cysts at 1 week (Dissen et al., 2001). Blocking two other neurotrophins, Neurotrophin 4 (NT4) and Brain-derived Neurotrophic Factor (BDNF), with antibodies in neonatal ovary organ culture caused a reduction in oocyte survival (Spears et al., 2003). Mutation of the receptor for
NT4 and BDNF, *neurotrophic tyrosine kinase receptor type 2* (*ntrk2*), also resulted in a reduction of oocytes. Recent studies of ovaries from homozygous mutants of *ntrk1*, encoding the receptor for NGF, as well as *ntrk2*, report a reduction in the number of oocytes enclosed in follicles at 1 week supporting the role of Neurotrophins in germ cell cyst breakdown and primordial follicle formation (Kerr et al., 2009).

Mutations in at least three different genes encoding transcription factors cause female infertility resulting from altered primordial follicle assembly and subsequent oocyte loss (Table 1.4). *forkhead box l2* (*foxl2*) encodes a winged helix transcription factor, and granulosa cells do not properly surround oocytes to form primordial follicles in mutant females (Schmidt et al., 2004; Uda et al., 2004). Many germ cells were still in germ cell cysts at 1 week though some follicles did form, and many dying oocytes were observed by 8 weeks. *newborn ovary homeobox-encoding gene* (*nobox*) mutants have a similar phenotype with many more germ cells still in cysts at 1 week after birth (Rajkovic et al., 2004). However, oocyte loss was even more pronounced than in *foxl2* mutants with most oocytes lost by 2 weeks after birth. The third transcription factor with a similar mutant phenotype is Factor in the germ line alpha (*figla*), also known as Folliculogenesis specific basic helix-loop-helix (Soyal et al., 2000). The *figla* phenotype was the most severe with no primordial follicles formed and loss of most oocytes by 1 week after birth.

Mutants have also been identified that form follicles at a faster rate than normal. Aryl hydrocarbon receptor (Ahr) is a basic helix-loop-helix protein. *ahr* mutants have accelerated follicle formation, though by 8 days after birth the number of follicle was the same as wild-type (Benedict et al., 2000; Robles et al., 2000). Another mutant with accelerated follicle formation is *p27*, which is also known as *cyclin-dependent kinase (cdk) inhibitor 1* (Rajareddy et al., 2007). The p27 protein also plays a role in follicle activation, as described in the next section.

According to several groups, bovine follicles begin to form at approximately 90 days of gestation (Dominguez et al., 1988; Russe, 1983; Yang & Fortune, 2008), although there is some controversy about the exact timing of follicle formation, with researchers observing primordial follicles as early as 74 days (Nilsson & Skinner, 2009; Tanaka et al., 2001) and one not observing follicles until 130 days (Erickson, 1966). As in rodents, follicle formation begins with the innermost region first (Russe, 1983). There is also a large number of apoptotic cells present as follicles are forming (Erickson, 1966; Garverick et al., 2010), which has been observed in most mammalian species that have been examined (Baker, 1972). Oocytes not surrounded by granulosa cells are thought to degenerate (Adams et al., 2008; Smitz & Cortvrindt, 2002).

In the sheep there is also some variation about the timing of follicle formation, with one study reporting the first follicles at 66 days and another at 75 days of gestation (Juengel et al., 2002; Russe, 1983). Similar to bovine primordial follicle formation, the first follicles that form are located at the interface of the ovarian cortex and medulla, and follicles form progressively toward the outer cortex (McNatty et al., 2000; Sawyer et al., 2002). A large number of germ cells, over 75%, undergo apoptosis as the follicles are forming (Sawyer et al., 2002; Smith et al., 1993). It is thought that one reason for oocyte death is that the loss of germ cells allows more pregranulosa cells to associate with an individual surviving oocyte. The pregranulosa cells also extend cyttoplasmic protrusions between the oocytes, which may help to separate oocytes in cysts (Sawyer et al., 2002).

There is less information available regarding follicle formation in other domestic animals. In the pig, follicles begin to form at approximately 56 days of gestation (Bielanska-Osuchowska, 2006). As with cows and sheep, the first follicles form in the deepest part of the ovary. Primordial follicles are first observed in the goat at 90 days of gestation (Pailhoux et al., 2002; Pannetier et al., 2006) and in the horse at 102 days (Deanesly, 1977).
1.5 Follicle Development

Primordial follicles, each consisting of an oocyte and several granulosa cells that exhibit a flattened shape, remain dormant for varying amounts of time until activated to grow (Figure 1.2). A change in the morphology of the granulosa cells from flattened to cuboidal is indicative of follicle activation, and at this point the oocyte and associated granulosa cells are referred to as a primary follicle (Figures 1.2b and 1.3c). The primary follicle is enclosed in a basal lamina (Aerts & Bols, 2010). The oocyte remains arrested in the diplotene stage of prophase I of meiosis as the follicle grows. In addition, during follicle growth, the oocyte itself also grows, and in the mouse increases in size 300-fold in a 2- or 3-week period (Lintern-Moore & Moore, 1979). Furthermore, RNA content increases by 300-fold and protein synthesis by 38-fold (Wassarman & Albertini, 1994). As the granulosa cells of primary follicles divide to produce multiple cell layers, secondary or preantral follicles are formed (Figures 1.2c and 1.3d). Theca cells, which form from fibroblast-like cells in the ovarian stroma, become associated with the follicles at this stage (Hirshfield, 1991). The theca and granulosa cells support the oocytes and also produce hormones (Erickson et al., 1985). The preantral follicles eventually gain a fluid-filled space, and are then classified as antral follicles (Figure 1.2d). Many follicles do not survive past this stage. The surviving follicles are termed preovulatory follicles. Meiotic arrest is released just prior to ovulation in response to gonadotropins (the LH surge) (Jamnongjit & Hammes, 2005). The oocyte proceeds through meiosis and is then arrested a second time, in metaphase II, until fertilization.

In the mouse, some follicles begin to develop almost immediately after forming and are referred to as the first wave of developing follicles (Hirshfield & DeSanti, 1995). These first follicles that form are located in the core of the ovary. The follicles reach the antral stage by 3 weeks after birth and then become atretic and die because there is no gonadotropic surge to rescue them (Mazaud et al., 2002; McGee et al., 1998; Rajah et al., 1992). It is unclear why this first wave of follicular development occurs.

Follicle activation and development can be divided into two phases: initial recruitment and cyclic recruitment (McGee & Hsueh, 2000). Initial recruitment is a continuous process referring to the activation of groups of primordial follicles. It is thought that there are inhibitory factors that suppress the activation of follicles, and a few inhibitory proteins have been identified so far (Adhikari & Liu, 2009). Little is known about the mechanisms that control the selection of follicles that are activated.
One idea is that the follicles are recruited in the same order as they entered meiosis (Edwards et al., 1977). This idea is supported by the observation that oocytes in the medullary or inner cortex region of the ovary enter meiosis first and it is in this region where the first developing follicles are observed. During preantral growth, the granulosa cells multiply, the oocyte grows, the zona pellucida forms, the theca layer is made, and a vascular supply develops (McGee & Hsueh, 2000). Communication between the oocyte and the surrounding granulosa cells is very important during this phase of follicle growth (Tsafri, 1997). This communication is in part through gap junctions connecting the oocyte to the granulosa cells.

The oocyte gains the ability to resume meiosis about the time the antrum begins to form in the follicle and is designated as meiotic competence (Mehlmann, 2005). At this point the oocyte
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has the required level of maturation-promoting factor (MPF) necessary to resume meiosis. MPF is a complex consisting of CDK1 and cyclin B. However, even though the oocyte is capable of resuming meiosis, meiotic arrest is maintained until the LH surge. Oocytes from antral follicles will spontaneously resume meiosis if removed from the follicle, and thus a signal from the granulosa cells is important. This signal results in high cAMP levels in the oocyte, which are important for maintenance of meiotic arrest.

Cyclic recruitment refers to the selection of only a few follicles to reach the preovulatory stage. Before puberty, follicles that grow eventually undergo atresia. After puberty, at the antral stage, only a few follicles continue to grow while the rest die. Both the oocyte and the granulosa cells of the atretic follicles undergo apoptosis (Pesce & De Felici, 1994). In response to FSH, one oocyte (or several if the species is polyovulatory) grows faster and becomes the dominant follicle (Zeleznik & Benyo, 1994). The dominant follicle then makes estrogen and inhibin, which suppresses FSH and inhibits the remaining follicles from growing. Dominant follicle selection has been most well studied in the cow and horse; it is discussed below.

Several genes have been implicated in the regulation of primordial follicle activation (see Table 1.5). One common mutant phenotype is the premature activation of all primordial follicles, leading to the eventual loss of all oocytes. The mice are initially fertile but eventually become sterile as the oocytes are lost. This suggests that this group of genes is involved in blocking primordial follicle activation. Several of the mouse mutations with this phenotype are associated with the phosphatidylinositol 3 kinase (PI3K) signaling pathway. One PI3K signaling mediator is 3-phosphoinositide dependent protein kinase 1 (PDPK1), which phosphorylates AKT serine/threonine kinases (Reddy et al., 2009). pdpk1 mutant females have a gradual loss of fertility due to the premature activation of follicles. However, akt1 mutants are less severe with reduced fertility and premature activation of only a subset of follicles (Brown et al., 2010). Another mutation where the primordial follicle pool becomes prematurely activated is phosphatase and tensin homolog deleted on chromosome 10 (pten), a negative regulator of PI3K signaling (Reddy et al., 2008). Finally, mutants in three genes that are downstream of PI3K signaling, foxo3a, p27, and ribosomal protein s6 (rps6) also prematurely activate all follicles and become sterile (Castrillon et al., 2003; Rajareddy et al., 2007; Reddy et al., 2009).

Premature loss of follicles is observed in mutants of at least two genes that have not been associated with the PI3K signaling pathway (Table 1.5). First, the transcription factor, FoxL2 (which plays a role in primordial follicle formation), is also important in regulating follicle activation (Schmidt et al., 2004; Uda et al., 2004). All follicles are activated by 2 weeks after birth in foxl2 mutants. Second, although anti-mullerian hormone (amh) mutant females are reported to be fertile, there is a premature reduction in the pool of primordial follicles (Durlinger et al., 1999).

Mutation of several transcription factors causes infertility with arrest at the primordial follicle stage and eventual oocyte depletion (Table 1.5). This phenotype suggests that these molecules are required for the activation of follicles and progression to the primary follicle stage. The gene encoding LIM homeobox protein 8 (Lhm8) when mutant causes arrest of follicles at the primordial follicle stage (Choi et al., 2008a; Pangas et al., 2006). Mutations in nobox also have follicles arrested at the primordial stage (Rajkovic et al., 2004). Two basic helix-loop-helix encoding genes, spermatogenesis and oogenesis-specific basic helix-loop-helix 1 (sohlh1) and sohlh2, also belong to this class of mutations that are arrested at the primordial follicle stage (Choi et al., 2008b; Pangas et al., 2006).

Another class of mutants has follicles that are activated but arrest at the primary follicle stage (Table 1.5). For example, gdf9 mutants cannot progress farther than the primary follicle stage and are therefore sterile (Dong et al., 1996). Similarly, some mutants in the receptor tyrosine kinase, kit
Table 1.5 Genes involved in follicle activation and early follicle development.

<table>
<thead>
<tr>
<th>Protein/Function</th>
<th>Female Mutant Phenotype</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>akt</td>
<td>Reduced fertility, premature reduction of primordial follicle pool.</td>
<td>(Brown et al., 2010)</td>
</tr>
<tr>
<td>amh</td>
<td>Fertile, premature reduction of primordial follicle pool.</td>
<td>(Durlinger et al., 1999)</td>
</tr>
<tr>
<td>foxl2</td>
<td>Progressive loss of fertility, premature activation of all follicles.</td>
<td>(Schmidt et al., 2004; Uda et al., 2004)</td>
</tr>
<tr>
<td>Foxo3a</td>
<td>Progressive loss of fertility, premature activation of all follicles.</td>
<td>(Castrillon et al., 2003)</td>
</tr>
<tr>
<td>gdf9</td>
<td>Sterile, arrest at primary follicle stage, oocyte loss.</td>
<td>(Dong et al., 1996)</td>
</tr>
<tr>
<td>kit</td>
<td>Some mutants arrest at primary follicle stage.</td>
<td>(Yoshida et al., 1997)</td>
</tr>
<tr>
<td>kitl</td>
<td>Some mutants arrest at primary follicle stage.</td>
<td>(Bedell et al., 1995)</td>
</tr>
<tr>
<td>lhx8</td>
<td>Sterile, arrest at primordial follicle stage, oocyte loss.</td>
<td>(Choi et al., 2008a; Pangas et al., 2006)</td>
</tr>
<tr>
<td>nobox</td>
<td>Sterile, arrest at primordial follicle stage, oocyte loss.</td>
<td>(Rajkovic et al., 2004)</td>
</tr>
<tr>
<td>p27</td>
<td>Progressive loss of fertility, premature activation of all follicles.</td>
<td>(Rajareddy et al., 2007)</td>
</tr>
<tr>
<td>pdk1</td>
<td>Progressive loss of fertility, premature activation of all follicles.</td>
<td>(Reddy et al., 2009)</td>
</tr>
<tr>
<td>pten</td>
<td>Progressive loss of fertility, premature activation of all follicles.</td>
<td>(Reddy et al., 2008)</td>
</tr>
<tr>
<td>rps6</td>
<td>Progressive loss of fertility, premature activation of all follicles.</td>
<td>(Reddy et al., 2009)</td>
</tr>
<tr>
<td>sohlh1</td>
<td>Sterile, arrest at primordial follicle stage, oocyte loss.</td>
<td>(Pangas et al., 2006)</td>
</tr>
<tr>
<td>sohlh2</td>
<td>Sterile, arrest at primordial follicle stage, oocyte loss.</td>
<td>(Choi et al., 2008b)</td>
</tr>
</tbody>
</table>

as well as in the kit ligand, stem cell factor (SCF), arrest at the primary follicle stage (Bedell et al., 1995; Dong et al., 1996; Yoshida et al., 1997). Interestingly, kit signaling can activate the PI3K signaling pathway, and this may be how kit regulates follicle development.

In most domestic species, primordial follicles do not develop into primary follicles immediately after forming, and there is a significant delay until the first appearance of primary follicles. In the cow, although primordial follicles form at 90 days of gestation, the first primary follicles are not observed until day 140 (Yang & Fortune, 2008). It is believed that these primordial follicles are not capable of activating directly after they form. It has also been observed that oocytes do not arrest in
the diplotene stage until approximately 141 days, suggesting that the oocyte must reach diplotene arrest before the primordial follicle can be activated to develop into a primary follicle.

In sheep, follicles start to form between 66 and 75 days gestation, and there seems to be some variability in the literature as to when they begin to develop (Juengel et al., 2002; Russe, 1983). The first developing follicles are observed at day 100 (Sawyer et al., 2002). Similarly, porcine follicles have been reported to form at 56, or 68 days of gestation, and the first developing follicles have been observed at 75 or 90 days (Bielanska-Osuchowska, 2006; Ding et al., 2010; Oxender et al., 1979).

The selection of the dominant follicle has been well studied in both bovine and equine species (Beg & Ginther, 2006; Fortune et al., 2004; Ginther et al., 2001). A wave of antral follicles is recruited to grow by a small increase in circulating FSH levels. After several days one of the follicles becomes larger than the other follicles and will likely become the dominant follicle while the other follicles become subordinate follicles and are eventually lost. The dominant follicle then synthesizes estradiol, and FSH levels decrease. It is believed that insulin-like growth factor (IGF) signaling may important for dominant follicle selection. Levels of free IGF are higher in the follicular fluid of the dominant follicle whereas IGF binding proteins that sequester IGF are low. In addition, in mice, igf1 mutants arrest by the early antral stage, suggesting that IGF is required for further development of the follicles (Baker et al., 1996).

The LH surge triggers the resumption of meiosis as well as other changes, including a change in mitochondrial localization. Prior to the LH surge, mitochondria are located peripherally in the oocyte, but during the final stages of nuclear maturation, they become more clustered (Ferreira et al., 2009). After ovulation, the mitochondria are dispersed throughout the cytoplasm. These changes are thought to reflect the changing energy requirements of the oocyte.

### 1.6 Steroid Hormone Signaling in Oocyte Development

Steroid hormone signaling is thought to be important for controlling the ability of bovine follicles to activate. Follicle activation can be blocked by exogenous estrogen treatment of cultured fetal bovine ovary explants (Yang & Fortune, 2008). In addition, fetal estrogen levels drop at about 141 days of gestation coinciding with follicle activation. Steroid hormone signaling has also been implicated not only in primordial follicle activation but also in primordial follicle formation in the cow. Nilsson and Skinner found that fetal ovarian estrogen and progesterone levels drop when primordial follicles begin to form (Nilsson & Skinner, 2009). They also showed that progesterone treatment of bovine ovaries in organ culture significantly blocked the assembly of follicles.

Fetal sheep ovaries produce both estrogen and progesterone (Lun et al., 1998). Steroidogenic cells have been identified in the ovine ovaries and high estrogen has been suggested to be important for ovigerous cord formation, while the drop in estrogen levels correlates with meiotic entry (Juengel et al., 2002).

Early rodent follicle development was thought to be independent of hormones, but recent work from several labs including ours suggests that steroids hormones are important in regulating germ cell cyst breakdown and primordial follicle formation (Chen et al., 2009; Chen et al., 2007; Kezele & Skinner, 2003; Lei et al., 2010). Adult female mice treated as neonates with estrogen or estrogen like compounds (Iguchi et al., 1990; Iguchi et al., 1986; Jefferson et al., 2002; Suzuki et al., 2002) have more MOFs suggesting that estrogen plays a role in controlling cyst breakdown and primordial follicle assembly (Gougeon, 1981; Iguchi & Takasugi, 1986; Iguchi et al., 1986). Our model is that normally, exposure of fetal oocytes to maternal estrogen keeps oocytes in cysts and at birth estrogen levels drop resulting in cyst breakdown. When oocytes are exposed to estrogens, cyst breakdown is
inhibited. Supporting this, our studies showed that during neonatal ovary development, mice treated
with the phytoestrogen, genistein, had more oocytes in cysts compared to control mice (Jefferson et al., 2006). Work from our lab has shown that estrogen causes a delay in individualization of oocytes, supporting the idea that MOFs are cysts that did not break down (Chen et al., 2007). Several synthetic compounds with estrogenic activity including bisphenol A, diethylstilbestrol, and ethylene estradiol also block cyst breakdown (Karavan & Pepling, 2012). Neonatal treatment with progesterone also results in more MOFs (Iguchi et al., 1988), and progesterone and estrogen affect neonatal oocyte development in rats (Kezele & Skinner, 2003). Neonatal progesterone treatment reduced primordial follicle assembly, while both progesterone and estrogen reduced primordial follicle activation. Interestingly, mutations in the orphan steroid hormone receptor, dosage sensitive sex reversal, adrenal hypoplasia critical region on chromosome X, gene 1 (dax) also have MOFs suggesting a role for the Dax protein in follicle formation (Yu et al., 1998).

In some species, estrogen seems to have a positive effect on follicle formation. In the hamster, estrogen promotes follicle assembly (Wang et al., 2008; Wang & Roy, 2007). In the baboon, if estrogen production is blocked, cyst breakdown and follicle formation are disrupted (Zachos et al., 2002). It is not clear why in some species estrogen promotes follicle formation and in other inhibits follicle formation. One possibility is that relatively high concentrations of estrogen inhibit follicle assembly while low concentrations promote assembly (Nilsson & Skinner, 2009). Alternatively there could be species differences in estrogen signaling.

1.7 Summary

Most mammals progress through similar stages of germ cell development, although there is wide
variation in the timing of each step. Some domestic animals are well studied, but information is
lacking on others. A more complete understanding of oogenesis in domestic species will lead to the
development of techniques to improve reproductive capacity and herd quality. Comparative studies
will also lead to a better understanding of human oogenesis and aid in treating infertility.

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over 100 kb 5′ of the Steel (Sl)-coding region in Steel-panda and Steel-contrast mice deregulate Sl expression and cause
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OOCYTE PHYSIOLOGY AND DEVELOPMENT IN DOMESTIC ANIMALS


OOCYTE DEVELOPMENT BEFORE AND DURING FOLLICULOGENESIS


Oocyte physiology and development in domestic animals


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