Morphogenesis and Female Fate Determination in the Vertebrate Ovary

by

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Dissertation submitted in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy in the Department of Cell Biology
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ABSTRACT
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Abstract

A unique feature of the fetal gonad is its ability to form two distinct organs, the testis and the ovary, from a single bipotential primordium. The outcome of this decision, which is made by a population of somatic cells known as the bipotential supporting cell precursors, determines whether an embryo will develop as a phenotypic male or female. Though several molecular pathways have been shown to be required for female fate determination in vertebrates, the intricacies of ovarian morphogenesis are not well understood. A key event in ovarian development occurs around birth, when meiotic germ cells and somatic granulosa cells organize into primordial follicles, the structures that generate mature oocytes for ovulation in adult females. We investigated the embryonic origins and proliferative properties of granulosa cells in the fetal mouse ovary and found that the precursors emerge from the ovarian surface epithelium and then enter mitotic arrest in a specification process that extends from the bipotential stage to the end of the postnatal follicle assembly period. Maintenance of cell cycle arrest in granulosa cell precursors appears to be regulated by Wnt signaling. The first granulosa cells to be specified were exclusively incorporated into the subset of follicles that begin to grow immediately upon assembly. We show that this first group of granulosa progenitors derives from the supporting cell precursors present in the bipotential gonad. Interestingly, both XX and XY supporting cell precursors are mitotically arrested towards the end of the bipotential period, indicating that adoption of supporting cell
fate might be regulated by the cell cycle. We present data suggesting that antagonism of Notch signaling is required for these precursor cells to exit the cell cycle and differentiate.

In Witschi’s classic model of vertebrate gonad development, the cortex and medulla of the undifferentiated gonad expand and differentiate in a mutually exclusive manner to yield the mature ovary and testis (Witschi 1951). Estrogen acts on both the cortex and medulla to promote female fate determination and ovary development in non-mammalian vertebrates. However, the downstream receptors and targets through which estrogen exerts its effects on the gonad have not yet been elucidated. We selected the red-eared slider turtle *Trachemys scripta* as a model with which to address this question. We first characterized the cellular composition of the turtle gonad before and after sex determination, identifying four populations of somatic cells distinguishable by their locations within the gonad as well as the complement of transcription factors expressed. Consistent with Witschi’s model, we determined that granulosa cell precursors arise from the surface epithelium of the turtle ovary after sex determination, similar to the timing of granulosa cell specification in the mouse. This morphological information was then applied to an investigation of estrogen signaling pathways in the turtle ovary. We show that i) estrogen likely acts through its canonical receptors rather than a non-canonical pathway involving ERK signaling; ii) early exposure to estrogen resulted in the premature downregulation of a testis-specific gene, *SOX9*, in the medulla; iii) less
estrogen is needed to promote expansion of the cortex than to repress testicular differentiation of the medulla, consistent with the localized production of estrogen in the medulla; and iv) estrogen’s repressive effect on SOX9 expression may be mediated by Wnt signaling.

Our findings add complexity to the standard model of how the male and female supporting cell lineages are established in mice, reveal evolutionary conservation between mice and turtles in the timing of granulosa cell specification relative to sex determination, and refine our understanding of how estrogen acts to promote ovarian development in non-mammalian species.
Dedication

I am deeply indebted to Drs. Kristi Judd, Ana Aparicio, and Alvaro Sagasti, who each, for reasons unbeknownst to me, hired me for research positions for which I lacked nearly all of the requisite skills and training and gave me the opportunity to learn at my own pace and figure out what it was that I really wanted to study in graduate school. My experiences in each of their labs awarded me with not only what felt to be a head start in graduate school but also the conviction this was the right career path for me.
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Acknowledgements

Walking in Duke’s Botanical Gardens one spring afternoon in 2007, a first year graduate student in search of a fourth rotation, I saw a group of mud-streaked pond turtles basking in the sun and thought to myself, wow, turtle embryos must be so adorable! This notion set off a cascade that would lead me directly to Blanche Capel’s lab at just the right time to take over her turtle project from a departing student, and, most importantly, to a group of labmates who would become like family to me, in a place that almost immediately felt like a home.

I am so grateful to Blanche for welcoming me into her lab, for giving me the freedom to explore the questions I found most appealing while guiding me towards interesting corollaries, for offering me a host of professional opportunities, and for frequently having more confidence in me than I had in myself. Making mouse chimeras with Blanche was definitely one of my favorite experiences in grad school.

I also want to thank Leo DiNapoli, who, as my rotation mentor, didn’t waste any time before starting to make fun of me, and thus, in kind of a twisted way, made me feel welcome right away. To Steve Munger, Matt Cook, Danielle Maatouk, Jonah Cool, Tony DeFalco, and Samantha Jameson – thanks to you all for being my lab family for so many years. You are all dear to me in your own ways, and this adventure wouldn’t have been nearly so much fun without you! Your various eccentricities, obsessions, predilections for pork, religious arguments, baby booms, and, of course, gonad jokes always made our
lab a very entertaining and tasty place to be. Thanks also to Iordan Batchvarov, who, despite his feigned gruffness, never hesitated to help me with any sort of problem and often went far out of his way to do so. To our newer lab members, Anirudh Natarajan, Michael Czerwinski, Jason Garness, Allison Navis, Ximena Bustamante, and Yi-Tzu Lin, I have greatly appreciated your enthusiasm and energy as my time at Duke winds up, and I hope you all have as much fun here as I did!

I am especially grateful to my committee, Peggy Kirby, Kathleen Smith, Donald McDonnell, and Fan Wang, for their excellent ideas, assistance, and encouragement over the years. I also want to acknowledge Doc Bonneau, my high school biology teacher, who inspired me to extend my love of the outdoors into a passion for biology.

I thank my parents, Jeff and Wendy Barske, and sister, Carrie, for expanding my horizons far beyond those of most of my childhood friends, for encouraging me to go to college on the other side of the country, and for their unquestioning support of my career choices. I have always felt exceptionally lucky to have been born into such a wonderful family.

And to my beloved husband, Nicolai Mork, who has willingly walked every step of this path with me, who has made a life with me here far away from our home in the west, who gives me encouragement, confidence, wisdom, pampering, and love every single day, I am infinitely grateful and forever indebted.
1. Introduction

Presumptive males and female vertebrate embryos are morphologically indistinguishable throughout early development. The first signs of sexual differentiation occur midway through development, when the initially bipotential gonads commit to the male or female pathway and begin to develop as testes or ovaries, in a process known as primary sex determination (Fig. 1). These organs quickly become morphologically and physiologically distinct from each other, despite their common origin and analogous function of protecting and propagating the germline. The germ cells are also initially bipotential. Regardless of their sex chromosome constitution, they begin to differentiate into sperm in a testicular somatic environment and into oocytes in an ovary (McLaren 1991). In eutherian mammals, secondary sex traits affecting the rest of the body generally become apparent only after the testis and ovary have diverged. The differentiating testis produces several hormones that masculinize the developing reproductive tract, external genitalia, and brain (Fig. 1). Ovarian hormones, in turn, are typically dispensable for the feminization of these structures but are required for mammary development after birth (Jost 1970; Dessauge et al., 2009).
Figure 1. Sexual differentiation begins with the gonads in eutherian mammals. XX and XY embryos are morphologically indistinguishable for the first half of development. The gonads (green) form at embryonic day (E) 10 and are bipotential until E11.5, such that they are capable of giving rise to either ovaries or testes. The Y chromosome-linked male sex-determining gene Sry drives testis development (blue) in XY embryos. In the absence of Sry, the gonads develop as ovaries (pink). By birth, hormones produced by the testis have induced the masculinization of the rest of the body, including the internal reproductive tract, external genitalia, and brain. In the absence of these hormones, female structures develop.
Figure 1: Sexual differentiation begins with the gonads in eutherian mammals.
1.1 Evolutionary divergence of sex-determining mechanisms

The signals that control the embryo’s decision of whether to develop testes or ovaries vary widely among vertebrates and are usually categorized as genetic mechanisms (GSD), where sex is inherited as a Mendelian trait, or environmental mechanisms (ESD), where a feature of the embryo’s external environment exerts a dominant influence over sex. GSD systems include male heterogamety (XX/XY), female heterogamety (ZZ/ZW), polygenic mechanisms, and homomorphic/cryptic sex chromosomes. ESD systems, in turn, include density-dependent mechanisms as well as temperature-dependent sex determination (TSD), where the embryo’s sex is sensitive to the incubation temperature of the egg. The methods of sex determination used by many species of reptile, amphibian and fish have been elucidated in recent years (Table 1) (Ryuzaki et al., 1999; Baroiller et al., 2001; Ogata et al., 2003; Janzen et al., 2006). When these are plotted onto a phylogenetic map, the evolutionary lability of sex determination is apparent within several major branches of the tree, where numerous transitions must have occurred to achieve the present diversity (Janzen et al., 2006).

Eutherian mammals, with XX/XY male heterogamety, comprise one of the most strictly GSD groups in the animal kingdom. The evolution of both viviparity and endothermy in eutherian mammals required a mechanism not based on temperature or the environment. Classic work in the mouse and human systems demonstrated that expression of the Y-chromosome-linked Sry gene leads to the differentiation of Sertoli
Table 1. Sex determination in extant vertebrates. Fish, amphibians, turtles, and lizards each exhibit more than one method of sex determination, which fall into genetic (GSD) and environmentally (ESD) based categories. XX/XY and ZZ/ZW refer to male and female heterogametic systems, respectively, while homomorphy refers to GSD in the absence of differentiated sex chromosomes. Polygenic and density-dependent sex determination have been observed in fish, and many reptiles and fish determine sex according to the incubation temperature of the egg (TSD). With the exception of eutherian mammals, most vertebrate embryos are susceptible to exogenous hormone-induced sex reversal. *Co-occurrence of TSD and GSD has been noted in several species of lizards. °Monotremes (i.e., platypus and echidna) have a complex arrangement of X and Y sex chromosomes, which assemble into a chain during meiosis. Modified from (Barske et al., 2009).
### Table 1: Sex determination in extant vertebrates

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GSD | ESD
cells and the adoption of testis fate (Gubbay et al., 1990; Lovell-Badge et al., 1995). In the absence of this upstream signal, ovarian development ensues. The accumulation of fertility factors on the Y chromosome and the low viability of XY oocytes (Lahn et al., 1997; Alton et al., 2008) operate to fix the GSD mechanism in mammals. However, viviparity and TSD coexist in several reptiles, where pregnant females can influence offspring sex ratio through their thermoregulatory behavior (Robert et al., 2001; Wapstra et al., 2004).

The red-eared slider turtle *Trachemys scripta* has been used as a model for TSD for several decades (Crews et al., 1991). In this species, embryos incubated at low temperatures during the thermosensitive period of development (TSP) invariably develop testes and become male, while embryos incubated at higher temperatures develop ovaries and become female (Bull et al., 1982). The pathway through which the temperature signal is translated into a morphogenetic event has not yet been elucidated.

Sex determination in non-eutherian vertebrate species is generally sensitive to exogenous sex hormones, especially estrogen (Table 1). For example, both marsupials, which share the *Sry*-dependent XX/XY system of eutherian mammals (Sharman et al., 1970; Foster et al., 1992) (though sex determination occurs after birth (Coveney et al., 2001)), and birds, which exhibit ZZ/ZW female heterogamety (which may depend on the dosage of the Z-linked *DMRT1* gene (Smith et al., 2009)), undergo various degrees of sex-reversal when exposed to hormones during the critical early period of gonad
development (Willier et al., 1935; Wolff et al., 1935; Coveney et al., 2001). Susceptibility
to exogenous hormone treatment is shared by most reptiles, including numerous species
traditionally classified as either GSD or TSD (Pieau 1970; Bull et al., 1988; Pieau et al.,
1994). More specifically, estrogen has been shown to be necessary and sufficient to
induce ovarian development in most non-mammalian vertebrates (Ramsey et al., 2008),
but it is completely dispensable for initial female fate determination in mice (Fisher et
al., 1998; Couse et al., 1999). The cell types and signaling pathways that mediate
estrogen’s ovary-promoting effect are unknown.

1.2 Establishment of the supporting cell lineage

The gonad contains a number of somatic cell types in addition to the germ cells. Of
these, the cells most intimately associated with the germline in adult testes and ovaries
are collectively referred to as the supporting cell lineages or respectively as Sertoli and
granulosa cells (Fig. 2). Sertoli cells line the inner surface of seminiferous tubules and
guide the development of spermatogonial stem cells into mature sperm. Granulosa cells,
also known as follicle cells, support the growth and development of oocytes inside
ovarian follicles. When a follicle is activated, the handful of granulosa cells surrounding
the single oocyte undergo numerous clonal divisions to produce the more than 2000
cells of the mature antral follicle (Hirshfield 1991). In both cases, the supporting cells and
germ cells are segregated away from the other cells in the gonad by a basement
membrane (Fig. 2).
Figure 2. Current model of the establishment of the male and female supporting cell lineages in mice. During the bipotential stage of gonad development (E10-11.5), a population of somatic cells underneath the coelomic epithelium are termed the bipotential supporting cell precursors (silver). These are the cells that express Sry and differentiate as Sertoli cells (blue) in the presence of a Y chromosome. The newly specified Sertoli cells quickly organize with germ cells (green) into testis cords, which become segregated from the rest of the cells in the testis by a layer of basement membrane (light grey). The fetal testis cords give rise to the seminiferous tubules in the adult, where Sertoli cells guide the differentiation of spermatogonial cells into mature sperm. The hormone-producing Leydig cells (yellow) lie outside the tubules. In the absence of Sry, the bipotential supporting cell precursors are thought to instead differentiate into granulosa cell precursors (pink). During folliculogenesis, which occurs shortly after birth, each oocyte (green) in the ovary becomes individually encapsulated by a dedicated set of granulosa cells, and the structure becomes lined by basement membrane. All of the granulosa cells in the large growing follicles present in the adult ovary stem from the handful of granulosa cells present at the primordial stage. The main hormone-producing cells of the ovary, the theca cells (yellow), are recruited to growing follicles from within the interstitium of the ovary.
Figure 2: Current model of the establishment of the male and female supporting cell lineages in mice.
In mice, the supporting cell lineages are thought to stem from a common progenitor population present in the bipotential gonad before the commitment to ovary or testis fate occurs (Fig. 2) (McLaren 1991; Albrecht et al., 2001). These supporting cell precursors are also the population in which primary sex determination takes place, at least in mammals (McLaren 1991). During the bipotential stage, the gonads are poised between testis and ovary fates, and “male” (e.g., Fgf9) and “female” (e.g., Wnt4) genes are expressed simultaneously (Kim et al., 2006). In XY embryos, the balance is tipped toward testis fate when the supporting cell precursors express the male sex-determining gene Sry. This gene upregulates a related gene, Sox9 (Sekido et al., 2008), which in turn sets off a cascade of male pathway genes that cause the cells to adopt Sertoli cell fate (reviewed by Kanai et al., 2005). Sertoli cells are thus the first cell type in the gonad to embark on a sex-specific fate. They subsequently instruct other gonadal cell types to adopt their own testis-specific fates and help to coordinate the organization of testis cords and recruitment of male-specific vasculature.

In XX embryos, which lack Sry, the testis pathway is suppressed, in part by canonical Wnt signaling (Tevosian et al., 2008), which permits activation of the female pathway and formation of an ovary. Although it is believed that the same supporting cell precursors that become Sertoli cells in XY gonads give rise to the granulosa cell population in adult XX individuals (McLaren 1991; Albrecht et al., 2001; Ito et al., 2006), this theory has not been confirmed. The instructive role of presumptive granulosa cells
in ovary development is also less clear than in males. Organization of germ cells and granulosa cell precursors into follicles, the functional unit of the ovary, occurs around birth in mice (Hirshfield 1991; Pepling et al., 2001), long after the primary sex-determining decision leads the bipotential gonad to embark on the ovarian pathway. Follicle assembly is known to depend on the presence of germ cells (in contrast to testis cord development (reviewed by McLaren 1991). While the signals that regulate each step of the follicle assembly process are not entirely clear, a reduction in estrogen signaling and activation of the Notch pathway have been implicated in the breakdown of oocyte nests into individual oocytes surrounded by a layer of granulosa cell precursors (Chen et al., 2007; Trombly et al., 2009).

The Sry-based model of how an upstream sex-determining signal is translated into a morphogenetic decision may not apply to all vertebrates, despite the noteworthy resemblance between the adult gonads of different species. Indeed, in zebrafish and medaka, the first cell type to show sex-specific differences is the germ cells, not the supporting cell precursors (Kobayashi et al., 2004; Tong et al., 2010). Another area of divergence lies in whether the male or female pathway is the “default fate” that the gonad adopts in the absence of the upstream signal that diverts the gonad to the alternate fate (e.g., Sry in mammals). For example, the female pathway is generally considered the default state in mammals, but in other species, the male pathway may be the default, with an active feminizing signal such as estrogen required for ovary
development. While this idea of a “default fate” is a rather simplified view of sex determination, it offers an interesting context in which to consider the contrasting effects of estrogen on sex determination in mammalian and non-mammalian vertebrates.

The work presented in this dissertation will clarify the origins of granulosa cells in the mouse ovary and reveal a new property of the bipotential supporting cell precursors that may be involved in their initial specification. The findings of a complementary project on the red-eared slider turtle reveal evolutionary conservation in the molecular identity of cell types present in developing mouse and turtle gonads and evaluate the potential involvement of a number of candidate pathways that might mediate the effect of estrogen on the turtle gonad. We highlight unexpected instances of similarity in how the male and female supporting cell lineages are established in mice as well as evolutionary conservation between mice and turtles in the timing of granulosa cell specification relative to sex determination.


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2. Materials and Methods

2.1 Mouse experiments

2.1.1 Mice, timed matings, genotyping, and tamoxifen administration

Tg(Sry-EGFP)92Ei transgenic mice (Sry-EGFP), in which EGFP expression is driven by a 5’ fragment of the Sry promoter, were kindly provided by K. Albrecht and E. Eicher (Albrecht et al., 2001) and maintained as homozygotes on the C57BL/6 background.

The Foxl2tm1(GFP/cre/ERT2)Pzg strain (Foxl2GCE), which carries a GFP-CreERT2 cassette knocked into the Foxl2 locus, was constructed by the GUDMAP consortium (Harding et al., 2011) and maintained on a C57BL/6 background. The presence of the mutant allele was determined on tail samples using primers that amplify Cre (Dietrich et al., 2000) or, to distinguish between heterozygotes and homozygotes, primers that target the Foxl2 locus: F (5’-AGAGAAGAGAGTGAGAGGGGTTTGC-3’), R1 (5’-GAGCGCCACGTACGAGTGAGCGCC-3’), and R2 (5’-GTCCAGCTCGACCAGGGGCGGT-3’). The PCR conditions were as follows: 94°C for 3 min, 35 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 45 sec, followed by 72°C for 10 min. The F/R1 primers amplify a 335-bp fragment of the wild-type allele, and the F/R2 primers amplify a 221-bp fragment of the mutant allele.

Gt(ROSA)26Sortm1Sor (R26R; (Soriano 1999)) mice were maintained on the C57BL/6 background and genotyped using primers that amplify lacZ.
Tg(Acta2-EYFP) transgenic mice (Acta2-EYFP), in which EYFP expression is regulated by a fragment of the smooth muscle alpha actin (Acta2) promoter, were provided by J. Lessard and maintained as homozygotes on an FVB; CD1 mixed background.

The Wt1tmCreER-IRESTMATOJq targeted line (Wt1CreER) was produced by J. Que and maintained on a CD1; C57CL/6 mixed background. The Tg(Nr5a1-cre)2Klp transgenic line (Sfl-Cre) was made by K. Parker (Bingham et al., 2006) and maintained on the C57BL/6 background. Both Wt1CreER and Sfl-Cre mice were genotyped using primers that amplify the Cre coding sequence.

The Gt(ROSA)26Sortm1(Notch1)Dam/J (RosaNICD) mice (Murtaugh et al., 2003) were kindly provided by D. A. Melton, maintained as homozygotes on a mixed 129; C57BL/6 background, and genotyped as needed using primers that amplify GFP.

Male Wt1CreER and Sfl-Cre mice were crossed to female RosaNICD mice in timed matings. In the Wt1CreER x RosaNICD cross, pregnant females were orally administered 1 mg tamoxifen/10 g body weight at E9.5 and E10.5 to induce Cre-mediated expression of the constitutively active intracellular domain of Notch1 fused to GFP. GFP expression was then monitored to determine which cells had been targeted (see section 4.3.2.1).

The floxed Numbtm1Ynj (Numbflor) strain (Zhong et al., 2000) and targeted null Numbtm1Wmz line (Petersen et al., 2002) were obtained from C. Kuo (Duke University) and maintained on a mixed background. The following primers were used for Numb and
Numbl genotyping: Numb flox F (5′-TAAGGATGTTTGTCTGCATGT-3′), Numb flox R (5′-TGAGTTACGTTCCCCTCACC-3′); Numbl 1 (5′-AGGGGCAGGCCACCATG-3′), Numbl 2 (5′-GTACCTGGGCACGTTGAAGT-3′), Numbl 3 (5′-CTTGGTCTTGTCCACCACCTC-3′), Numbl 4 (5′-ACGGGATCCCCCGGTATCGATAAGC-3′). The PCR conditions for both reactions were as follows: 95°C for 5 min, 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, followed by 72°C for 4 min. The wild-type Numb allele appears as a 120-bp fragment, while the floxed allele amplifies a 200-bp fragment. The wild-type Numbl allele is amplified as a 250-bp fragment, whereas the mutant allele appears as a 100-bp fragment.

The CAG-CreERT2 (ERCre) transgenic line (Hayashi et al., 2002) was maintained on a C57BL/6 background. Genotyping was performed on tail samples using previously published primers for Cre (Dietrich et al., 2000).

The transgenic Notch reporter line Tg(Cp-EGFP)25Gaia/J (TNR; (Duncan et al., 2005)) was maintained on a CD1 background in the homozygous state and genotyped as needed using primers for GFP.

The Numb^flox and Numbl^+/− lines were intercrossed with the R26R and TNR lines and maintained as Numb^flox/flox; Numbl^+/−; R26R; TNR or Numb^flox/flox; Numbl^+/−; R26R; TNR. The ERCre allele was crossed onto the Numb/Numbl line to generate stud males with the genotype ERCre; Numb^flox/flox; Numbl^+/−; R26R; TNR or ERCre; Numb^flox/flox; Numbl^+/−; R26R; TNR. Due to the reduced fertility exhibited by Numbl^+/− females, these males were crossed
with Numb\textsuperscript{flox/flox}; Numbl\textsuperscript{+/-}; R26R; TNR females in timed matings to produce XX and XY ERCre; Numb\textsuperscript{flox/flox}; Numbl\textsuperscript{+/-}; R26R; TNR embryos. Pregnant females were orally administered 1 mg tamoxifen/10 g body weight at E9.5 and E10.5 to induce Cre-mediated deletion of Numb and activation of the R26R reporter (see section 4.3.2.1).

The Fgf9\textsuperscript{tm1Dor} targeted null (Fgf9; (Colvin et al., 2001)) strain was maintained in the heterozygous state on the C57BL/6 background and genotyped using previously published primers (Colvin et al., 2001).

The 129-Wnt4\textsuperscript{tm1Amc/J} targeted null (Wnt4; (Vainio et al., 1999)) and the Ctnnb1\textsuperscript{tm1Mmt} (\(\beta\)-catenin\textsuperscript{ex3}; (Harada et al., 1999)) strains were maintained on a C57BL/6 background and genotyped using previously published primer sets ((Stark et al., 1994) and (Harada et al., 1999), respectively). Male Sf1-Cre mice were crossed to \(\beta\)-catenin\textsuperscript{ex3} females in timed matings to obtain female embryos in which \(\varepsilon\)-catenin was stabilized in the somatic cells of the gonad (Maatouk et al., 2008) (see section 4.3.2.5).

The Tg(BRE-Hspa1a-lacZ)1C10Ox (BRE-lacZ) transgenic line, in which lacZ expression is driven by a series of Smad1/5/8 binding sites from the Id1 promoter (Blank et al., 2008), was maintained on an outbred genetic background and genotyped using primers for lacZ. This line was crossed to the Foxl2\textsuperscript{GCE} and Wnt4 mutant lines to generate BRE-lacZ; Foxl2\textsuperscript{GCE+} and BRE-lacZ; Wnt4\textsuperscript{+/-} stud males, respectively, which were then crossed back to Foxl2\textsuperscript{GCE+} or Wnt4\textsuperscript{+/-} females to evaluate BMP signaling activity in the absence of Foxl2 or Wnt4. We also generated BRE-lacZ; Foxl2\textsuperscript{GCE+}; Wnt4\textsuperscript{+/-} animals, which
were then intercrossed to produce double mutant embryos carrying the BRE-lacZ reporter (see section 8.3.2).

The Rosa26\textsuperscript{CAG-loxptoplox-caBmpr1a} (Bmpr1a\textsuperscript{CA}) strain (Rodriguez et al., 2010), in which a constitutively active BMPR1A protein is expressed upon Cre-mediated excision, was maintained on an outbred background and genotyped using the following primers: caBMPR1A Rosa/01F (5′-CACTTGCTCTCCAAAAGTCG-3′), caBMPR1A Rosa/02B (5′-TAGTCTAACTCGCGACACTG-3′), and caBMPR1A CAG/02B (5′-GGTATGTAACGCGGAACTCC-3′). The PCR conditions were as follows: 96°C for 3 min, 36 cycles of 96°C for 30 s, 60°C for 30 s, and 72°C for 1 min, followed by 72°C for 10 min. The wild-type band is 300 bp, and the mutant band is 520 bp. Homozygous Bmpr1a\textsuperscript{CA/CA} males were crossed to Sf1-Cre females in timed matings to induce constitutive BMP signaling in the somatic cells of the gonad (see section 8.3.3).

Mice expressing EGFP from the β-actin promoter and CMV intermediate early enhancer (FVB.Cg-Tg(CAG-EGFP)B5Nagy/J) were maintained as homozygotes on the FVB background.

Outbred CD1 animals were used to establish the time course of cell cycle arrest (see section 3.3.2) as well as for making chimeras (see section 2.1.7) and performing BrdU and MitoTracker lineage tracing experiments and (see section 3). To determine the XY genotype of assorted mutants and embryos younger than E12.5, tail DNA samples were subjected to PCR using primers that detect Kdm5c (X chromosome) and Kdm5d (Y
chromosome) (Mroz et al., 1999). For timed matings, females were inspected each 
morning for the presence of a vaginal plug, and noon on the day that a plug was 
observed was designated embryonic day (E) 0.5. All mice were housed in accordance 
with NIH guidelines, and experiments were conducted with the approval of the Duke 
University Medical Center IACUC.

2.1.2 Foxl2 lineage-tracing

To lineage trace Foxl2-expressing cells throughout development, Foxl2GCE/+ males 
were crossed to females carrying the R26R reporter, and pregnant females were injected 
intraperitoneally with 2 mg tamoxifen (20 mg/ml) per 40 g body weight at E12.5 or 
E14.5. Embryos were allowed to develop to E14.5 or postnatal day (P)7, P9, or P14 before 
dissection. At the indicated stages, pregnant females and pups were euthanized, and 
gonads were carefully removed and fixed for 30-45 min at room temperature or 
overnight at 4°C (see section 3.3.1).

2.1.3 Mouse gonad culture

Fetal mouse gonads were cultured using the agar block method (Martineau et al., 
1997). Gonads were cultured in grooves cast in small gels made of 1.5% agar in 
Dulbecco’s modified eagle medium (DMEM). The blocks were placed in 35-mm culture 
dishes and bathed in DMEM containing 10% fetal bovine serum (FBS) and 50 μg/ml 
ampicillin and cultured at 37°C with 5% CO2. The medium was removed from the wells
so that the gonads remained fairly dry. Tissues were moistened with media each day of the culture period.

2.1.3 MitoTracker labeling

Gonads were dissected from embryos/pups at stages E11.5-14.5, P1, P3, or P7 and cultured using the agar block method. MitoTracker Orange CMTMRos (Invitrogen) was diluted in culture medium to a final concentration of 1 µM and then applied to the gonad surface with a pipette. The dye was washed off after 30 min at 37°C, and samples were cultured for 2-96 h at 37°C with 5% CO₂, and then fixed in 4% paraformaldehyde for 45 min at room temperature (see sections 3.3.3 and 3.3.4).

2.1.3.2 Endothelial cell depletion

To test whether endothelial cells stimulate Sertoli cells to resume cell cycle, we inhibited vascular development in XY gonads by one of two methods. The first method involved su5402, an inhibitor of multiple tyrosine kinase receptors (Mohammadi et al., 1997). We observed that exposure to this drug induces widespread endothelial cell depletion. E11.5 XY gonads were cultured in agar blocks containing 10 µM su5402 for 24 h, fixed in 4% paraformaldehyde, and immunostained. The second method involved VEGF trap (aflibercept; Regeneron), a molecule that binds and inhibits extracellular VEGF and prevents endothelial migration into the gonad (Cool et al., 2011). Following Cool et al. (2011), the drug was introduced into the circulation of XY embryos, and the
gonads were subsequently cultured using the agar block method for 28 h, fixed in 4% paraformaldehyde, and immunostained (see section 4.3.3.1).

2.1.3.3 FGF9 bead culture

Beads were coated with FGF9 or bovine serum albumin (BSA) and placed next to E11.5 gonads cultured in agar blocks, as previously described (Kim et al., 2006). Samples were cultured for 24 h, fixed in 4% paraformaldehyde, and immunostained (see section 4.3.3.2).

2.1.3.4 Retinoic acid culture

To determine whether exogenous retinoic acid could prevent Sertoli cells from reentering cell cycle, E11.5 gonads were cultured for 24 h in agar blocks soaked in 1 μm retinoic acid or dimethylsulfoxide (DMSO; control), fixed in 4% paraformaldehyde, and immunostained (see section 4.3.3.3).

2.1.4 Busulfan injections

Pregnant CD1 females were injected intraperitoneally at E10.5 with either 10-30 mg of busulfan (Sigma) dissolved in 50% DMSO or an equivalent volume of 50% DMSO (see section 4.3.2.2).

2.1.5 BrdU tracing and quantitation

Pregnant females were injected intraperitoneally with 1.5 mg 5-bromo-2'-deoxyuridine (BrdU; Sigma) dissolved in 7 mM NaOH/phosphate-buffered saline (PBS) at stages E11.5-14.5. Pups were subcutaneously injected at P1 or P4 with 50 μg BrdU/g
body weight. Two hours post-injection, embryos/pups were either dissected or injected with excess thymidine (25 mg) and allowed to develop for 24-48 h (embryos) or 3-6 days (pups) before dissection. Alternately, dissected gonads were cultured in medium supplemented with 3.125 μg/ml BrdU for 1 h at 37 °C prior to fixation. Gonads were fixed in 30% 50 mM glycine/70% ethanol or 4% paraformaldehyde for 1 h at room temperature. Samples were washed once in PBS, treated with 2 M HCl for 30 min at room temperature, washed again, and then subjected to immunocytochemistry as described below. To estimate the proportion of BrdU/FOXL2 double-positive cells in the total FOXL2-positive population, gonads were immunostained with antibodies against BrdU and FOXL2 and imaged at 40× magnification on a LSM710 Meta confocal microscope (Carl Zeiss, Inc.). Images (2-3 per sample) were taken near the center of each gonad, with 2-4 gonads imaged per time point. For each image, the numbers of FOXL2-positive and BrdU/FOXL2 double-positive cells were carefully counted in Adobe Photoshop and expressed as a percentage. For each injection stage, we compared the proportions of BrdU/FOXL2 double-positive cells after 24- and 48-h traces using a two-tailed Student’s t-test. The data are presented as the mean ± SEM (see section 3.3.3).

2.1.6 X-gal staining

Gonads from BRE-lacZ embryos and prepubertal females were fixed for 5 min at room temperature in 4% paraformaldehyde, rinsed, and washed for 5 min in a wash solution made with 0.4 ml of 1 M MgCl₂, 2 ml of 2% NP-40, and 197.6 ml PBS. Samples
were then incubated overnight at 37°C in a standard X-gal staining solution and imaged the following day with a Leica DFC300 camera (see sections 8.3.1 and 8.3.2).

2.1.7 Production of chimeras

Chimeras were produced between embryos derived from wild type CD1 x CD1 matings and embryos derived from matings between CD1 and mice expressing EGFP from the β-actin promoter and CMV intermediate early enhancer (FVB.Cg-Tg(GFPU)5Nagy/J (EGFP)). Embryos were flushed from the oviducts of pregnant females on embryonic day E2.5. The zona pellucida was removed, and embryos were assembled in pairs (one from each mating) in shallow wells, cultured overnight to the blastocyst stage, then transferred to E2.5 pseudopregnant female hosts (Nagy et al., 2003) (see section 9.3.1).

2.1.8 Fluorescence activated cell sorting

Liver tissue was incubated in 1 ml of Trypsin-EDTA (1X) at 37°C for 15-20 min and passed through a 27.5 gauge syringe four times to dissociate the tissue and separate the clumps of cells. Cells were centrifuged at 5000 rpm for 3 min, washed two times in PBS, resuspended in 500 µl of PBS, placed on the filter of a 5-ml glass conical tube (BD Falcon REF 352235), and briefly centrifuged. GFP-positive and negative cells were sorted using the Beckman-Coulter MoFlo or FACS Vantage Flow Cytometry Shared Resource (Duke Comprehensive Cancer Center) (see section 9.3.1).
2.2 Turtle experiments

2.2.1 Turtle eggs

Freshly laid red-eared slider turtle eggs were obtained from the Kliebert and Clark turtle farms (Hammond, LA) with the approval of the Louisiana Department of Agriculture and Forestry. Eggs were incubated in moist vermiculite in humidified incubators at 26°C (male-producing temperature, MPT) or 31°C (female-producing temperature, FPT) with ambient CO₂. Incubation at these temperatures yields 100% male and 100% female embryos, respectively (Bull et al., 1981; Wibbels et al., 1991). Embryos were staged using criteria established by Greenbaum (Greenbaum 2002). The temperature-sensitive period extends from the onset of gonad development (~stage 14) to stage 19 at the FPT and stage 20 at the MPT (Wibbels et al., 1991), and lasts for ~9-25 days, depending on the incubation temperature.

At stages of interest (e.g., 15, 17, 18, 19, 21 and 26), embryos incubated at the MPT or FPT were removed from eggs, decapitated, and placed into PBS for dissection. Gonad-mesonephros complexes (GMCs) were dissected from each embryo and fixed in 4% paraformaldehyde overnight at 4°C. Samples were then either immunostained immediately, dehydrated through a methanol gradient and stored in 100% methanol at -80°C, or dehydrated through a sucrose gradient and embedded in OCT for cryosectioning.
2.2.2 β-estradiol, letrozole, and ICI182780 treatments

17β-estradiol (E2; Sigma; 5 μg in 10 μl 95% ethanol) was administered to eggs incubating at the MPT, and the non-steroidal aromatase inhibitor letrozole (Novartis; 10 μg in 10 μl 95% ethanol) was administered to eggs incubating at the FPT. The drugs were topically applied to the eggshell in the region adjacent to the embryo (Crews et al., 1991) at stages 13, 15, 17 and 19 (see sections 6.3.4 and 6.3.5).

For late-stage estrogen treatments, eggs incubating at the MPT were given one dose of 5 μg E2 at stage 23 and a second dose four days later, then were dissected at stage 25 (six days after the first treatment) (see section 6.3.5).

To inhibit estrogen signaling, the estrogen receptor (ER) antagonist ICI182780 (Sigma) was topically applied to eggs incubating at the FPT at stages 17, 19, and 20, at a dose of 55.6 μg in 10 μl 95% ethanol. This dose of ICI182780 is the molar equivalent of a 25 μg dose of E2. GMCs were dissected from treated embryos at stages 21 and 26. In all experiments, controls were treated with 10 μl of 95% ethanol (see section 6.3.2).

2.2.3 Turtle gonad culture

Two similar culture methods for turtle gonads have been reported (Moreno-Mendoza et al., 2001; Shoemaker-Daly et al., 2010). We designed a third method, modified from (Martineau et al., 1997), with the aim of improving morphology, facilitating tissue access to inhibitors, and reducing contamination. Gonads were separated from the adjacent mesonephros prior to culture. Isolated gonads were laid in
thin wells shaped in strips of agar gel (1.5% agar in Leibovitz’s L-15 medium (Gibco); approximately 0.3 ml in volume) placed in 35-mm tissue culture dishes. Each strip was bathed in 0.3 ml of culture medium comprised of 10% charcoal-stripped FBS in Leibovitz with 50 μg/ml ampicillin and 1.25 μg/ml Fungizone (Gibco). Excess medium was removed from the wells so that the gonads remained fairly dry. The samples were cultured at FPT or MPT in ambient CO₂ for six to ten days. Approximately 5 μl of medium was pipetted directly onto the gonads and incubated for ~5 min on each day of the culture period. The culture medium was changed every other day. LiCl (25 mM final concentration), letrozole (1-2 μM in ethanol; Sigma), GSK-XV (5 μM in DMSO), XAV939 (2 μM in DMSO; a kind gift from F. Cong), IWR-1 (10 μM in DMSO; Sigma), or aphidicolin (3.75 μg/ml in DMSO; Calbiochem) was added to the culture medium for the respective experiments. Controls were exposed to equivalent concentrations of solvent. At the end of the culture period, samples were washed in PBS, fixed in 4% paraformaldehyde at 4°C overnight, and immunostained.

Using this technique, we cultured stage 17 gonads for up to ten days. At this point, embryos incubating in ovo would have reached stage 21 (FPT) or stage 19/20 (MPT), though the progress of development in culture is not thought to mirror that observed in ovo (Shoemaker-Daly et al., 2010). Longer cultures were not attempted in this experimental series.
2.2.4 BrdU tracing

Gonad-mesonephros complexes were dissected from three to four FPT and MPT embryos at stages 18-22. Each complex was placed into a 40-μl droplet of culture medium supplemented with 3.125 μg/ml BrdU and cultured for 1 h at MPT or FPT. Samples were then washed in PBS, fixed in 4% paraformaldehyde overnight at 4°C, washed in PBS, exposed to 2 M HCl for 30 min at room temperature, washed again, and then subjected to immunocytochemistry. FPT gonads cultured with aphidicolin were transferred to droplets at the end of the culture period and exposed to BrdU as above (see section 5.3.3).

2.2.5 Short-term E2 and estriol cultures

GMCs were dissected from MPT embryos (stages 17, 18, or 19) and placed into droplets of Leibovitz L-15 medium containing in 10% charcoal-stripped FBS and 50 μg/ml ampicillin. E2 or estriol was then added to the culture medium to a final concentration of 1-500 ng/ml. Controls were exposed to equivalent concentrations of ethanol. The cultures were incubated at the MPT for 1-2.5 h, fixed overnight in 4% paraformaldehyde, and then immunostained for phosphorylated ERK as described below (see section 6.3.3).
2.2.6 Bright field imaging

To demonstrate the presence and absence of a blood-filled vascular network in the hatchling testis and ovary, respectively, newly dissected tissues were photographed with a Leica DFC300 camera (see section 5.3.4).

2.2.7 RNA isolation and quantitative RT-PCR

Gonads from embryos incubating at the FPT or MPT (+/– E2) were dissected away from the mesonephros (Ramsey et al., 2007), and individual pairs were stored in RNAlater at -80°C. RNA was extracted from each pair using TRIzol (Invitrogen) according to the manufacturer’s instructions, with the addition of 7.5 μg glycogen (GlycoBlue, Ambion) to each reaction before isopropanol precipitation. RNA was eluted with 8 μl DEPC-treated water, and genomic DNA contamination was removed by a 15-min room temperature DNase treatment (Sigma), followed by DNase inactivation with 2 μl DNase I Stop Solution at 70°C for 10 min. RNA was converted into cDNA using the BioRad iScript cDNA synthesis kit according to the manufacturer’s guidelines. WNT4 and SOX9 transcripts were amplified in triplicate from two to five pairs of gonads per sex/stage using the SensiMixPlus SYBR & fluorescein kit (Bioline) on an iCycler iQ Real Time PCR System (BioRad) as previously described (Shoemaker et al., 2007; Shoemaker et al., 2007). The primers used to amplify the turtle WNT4 transcript were previously reported (Shoemaker et al., 2007). SOX9 was amplified with forward 5’-CCTGCCCTTCTGGTCCG-3’ and reverse 5’-TCCTCGTCGCTCTTTTCTTCAG-3’
primers. The amplicon was sequenced, and melt curves were run for each sample to ensure amplification specificity. *PP1* was used as a normalizing gene (Shoemaker et al., 2007). PCR efficiencies for each gene were calculated from standard curves. The PCR cycling conditions were 95°C for 15 min, 45 cycles of 95°C for 30 s, 62°C for 30 s, 72°C for 30 s, then 72°C for 5 min. Mean normalized expression values were calculated as previously described (Pfaffl 2001; Simon 2003; Shoemaker et al., 2007), and significance (p < 0.05) was assessed using a two-tailed t-test (see sections 6.3.5 and 6.3.6).

### 2.3 Immunocytochemistry

Embryonic tissues were processed for whole-mount immunocytochemistry as follows. Fixed gonads were washed twice in PBS with 0.01% Tween-20 and incubated for 1 h at room temperature in a blocking solution consisting of 10% FBS, 3% BSA, and 0.2% Triton-X-100 in PBS. Samples were then incubated with primary antibodies (listed below) in fresh blocking solution overnight at 4°C. The next day, samples were washed three times for 30 min each in a washing solution made with 1% FBS, 3% BSA, and 0.2% Triton-X-100 in PBS and then blocked for 1 h before secondary antibodies (listed below) were applied. Samples were again incubated overnight at 4°C, washed three times the next day, and mounted in 1,4-diazabicyclo[2.2.2]octane (DABCO). Gonads were imaged in the longitudinal plane with a LSM710 Meta confocal microscope and the affiliated Zen software (Carl Zeiss, Inc.).
Postnatal samples were transferred through a sucrose gradient, embedded in OCT, and cryosectioned at 20-25 μm. For aromatase staining, stage 24 turtle gonad-mesonephros complexes were embedded in paraffin, sectioned at 5 μm, dewaxed, and subjected to sodium citrate-based antigen retrieval using standard methods. For both cryo- and paraffin sections, samples were subsequently rehydrated in PBS, blocked for 1 h, incubated with primary antibodies overnight at 4°C, washed three times for 10 min each, incubated with secondary antibodies for 1 h at room temperature, washed three more times, and then mounted in DABCO and imaged as above.

The primary antibodies used in these projects targeted aromatase (Abcam ab18995; 1:100), β-catenin (Sigma C7207; 1:200), β-galactosidase (MP Biomedicals 55976; 1:10,000), BrdU (Accurate Chemical OBT0030G; 1:200), CAVEolin (CAV1; BD Transduction Laboratories 610059; 1:200), DMRT1 (a kind gift from D. Zarkower (University of Minnesota); 1:500), E-cadherin (Invitrogen 131900; 1:250), FOXL2 (goat anti-FOXL2 from Novus Biologicals (NB100-1277; 1:200) or rabbit anti-FOXL2 (a kind gift from D. Wilhelm (University of Queensland); 1:500), GATA4 (Santa Cruz Biotechnology sc-1237; 1:100), GFP (Aves lab GFP1020, 1:500), phospho-Histone H3 (Cell Signaling 9710S; 1:500), H3K27 (Millipore 07-449; 1:150), Ki67 (NeoMarkers RM-9106-S; 1:500), α-laminin (a kind gift from H. Erickson (Duke University); 1:500), LHX9 (a kind gift from T. Jessell (Columbia); 1:500), phospho p44/42 MAPK (pERK, Cell signaling 9101S; 1:750), p21 (Santa Cruz Biotechnology sc-397G; 1:50), p27 (Santa Cruz Biotechnology sc-528; 1:500),
PECAM-1 (BD 553370; 1:500), SCP3 (Novus Biologicals NB300-231; 1:300), SOX9 (Chemicon AB5535; 1:1000), TEX14 (a kind gift from Martin Matzuk; 1:1000), mouse VASA homolog (MVH; Abcam ab13840; 1:500), and WT1 (Santa Cruz Biotechnology sc-192; 1:100). We used Cy2-, Cy3-, Cy5-, DyLight488-, AlexaFluor 488, or AlexaFluor 647-conjugated donkey or goat anti-rabbit, anti-rat, anti-mouse and anti-goat secondary antibodies (Jackson Immunoresearch and Invitrogen, respectively) at 1:250 or 1:500 dilutions to reveal primary antibody staining. Nuclei were stained with syto13 (Invitrogen SKU no. S7575, 1:1000), Hoescht 333420 (Invitrogen), or DAPI as needed.
3. Temporal differences in granulosa cell specification in the mouse ovary reflect distinct follicle fates

3.1 Summary

The embryonic origins of ovarian granulosa cells have been a subject of debate for decades. By tamoxifen-induced lineage tracing of Foxl2-expressing cells, we show that descendants of the bipotential supporting cell precursors in the early gonad contribute granulosa cells to a specific population of follicles in the medulla of the ovary that begin to grow immediately after birth. These precursor cells arise from the proliferative ovarian surface epithelium and enter mitotic arrest prior to upregulating Foxl2. Granulosa cells that populate the cortical primordial follicles activated in adult life derive from the surface epithelium perinatally and enter mitotic arrest at that stage. Ingression from the surface epithelium dropped to undetectable levels by postnatal day 7, when most surviving oocytes were individually encapsulated by granulosa cells. These findings add complexity to the standard model of sex determination in which the Sertoli and granulosa cells of the adult testis and ovary directly stem from the supporting cell precursors of the bipotential gonad.

3.2 Introduction

Granulosa cells play a critical role in supporting the growth and development of oocytes in immature and adult ovarian follicles. When a follicle is activated, the handful of granulosa cells surrounding the oocyte undergo at least ten clonal divisions to
produce the more than 2000 cells of the mature antral follicle (Hirshfield 1991). Some primordial follicles begin to grow shortly after assembly, but most lie dormant until they are activated during adult estrus cycles. The embryonic origin of granulosa cells is still not entirely clear. In mice, granulosa cells and Sertoli cells, the analogous supporting cell type in the testis, are thought to stem from a common progenitor population present in the bipotential gonad before the commitment to ovary or testis fate occurs (McLaren 1991; Albrecht et al., 2001). Sex determination in mammals is governed by an early fate decision in this cell population (McLaren 1991). In the presence of a Y-chromosome, these cells express the male sex-determining gene Sry, adopt Sertoli cell fate, and instruct the subsequent development of the testis. Although it is believed that these bipotential precursor cells give rise to the granulosa cell population in adult XX individuals, this theory has not been confirmed.

Ultrastructural studies of diverse mammalian ovary models originally posited three potential sources of granulosa cells: the aforementioned supporting cell precursors in the bipotential gonad, the ovarian surface epithelium, and the rete ovarii at the ovary-mesonephros border (reviewed by Auersperg et al., 2001; Liu et al., 2010). The first two cell types are likely directly related, as a previous cell labeling study showed that at least some of the supporting cell precursors in the early male gonad derive from the surface epithelium (Karl et al., 1998). No study has definitively shown that cells actively enter the ovary from the rete ovarii. It is possible that granulosa cells derive from more than
one of these populations or that their origins differ between species. In rodents, though perhaps not in all mammals, it is thought that a restricted population specified during development accounts for all of the granulosa cells in the definitive pool of primordial follicles that is assembled shortly after birth (Hirshfield 1991; Chang et al., 2008). This model would be parallel to the situation in males, where pre-Sertoli cells specified during fetal life populate testis cords and give rise to the entire population of adult Sertoli cells (Sekido et al., 2004).

One line of evidence that granulosa and Sertoli cells arise from a common bipotential progenitor came from the observation that granulosa cells can transdifferentiate into Sertoli-like cells and vice versa in several different rodent models, most of which involved a loss of oocytes or estrogen deficiency (McLaren 1991; Couse et al., 1999; Britt et al., 2002; Matson et al., 2011). A second piece of evidence for the common origin hypothesis was provided by Albrecht and Eicher (2001), who generated a transgenic mouse line in which EGFP was driven by a partial Sry promoter (Sry-EGFP) (Albrecht et al., 2001). In XY transgenic embryos, the EGFP expression pattern resembled endogenous Sry expression, but XX gonads also expressed EGFP in a subpopulation of somatic cells, indicating that these cells were competent to activate the Sry promoter. Based on these results, the authors proposed that cells capable of activating the Sry promoter represent a common bipotential precursor population for the supporting cell lineage from which both Sertoli and granulosa cells are derived. They noted that a few
granulosa cells retained reporter activity perinatally (Albrecht et al., 2001), but it was unclear whether these cells were the direct descendants of the earlier EGFP-expressing precursors or had activated the promoter *de novo*. To address this issue, Ito et al. (2006) traced the developmental outcome of XX cells that activated an *Sry-Cre* (constructed with different promoter regions) and detected positive reporter activity two weeks after birth in a small subset of granulosa cells, but no other cell types (Ito et al., 2006). Though these results are more definitive, the fact that only a few granulosa cells were labeled left open the possibility that other cell types contribute to the granulosa cell population in the adult ovary before or after birth.

*Foxl2* is a forkhead transcription factor expressed in somatic cells of the early XX gonad (Schmidt et al., 2004). In goats, a female-to-male sex reversal phenotype associated with polled intersex syndrome has been attributed to misregulation of *FOXL2* expression (Pailhoux et al., 2005; Boulanger et al., 2008). These findings led to the speculation that *FOXL2* is an ovary-determining gene, parallel to *SRY* in males. However, in humans, mutations in *FOXL2* do not lead to sex reversal, but rather to blepharophimosis-ptosis-epicanthus inversus syndrome (BPES), which results in eyelid malformations and premature ovarian failure (Crisponi et al., 2001). *Foxl2* is likewise not required for the initial determination of ovarian fate in mice, as null mutants do develop ovaries (Schmidt et al., 2004; Uda et al., 2004), but these mutant ovaries upregulate components of the testis pathway during late embryonic development (Garcia-Ortiz et
al., 2009), and postnatal follicle activation is severely impaired (Schmidt et al., 2004; Uda et al., 2004). Moreover, deletion of Foxl2 in the adult mouse ovary led to a loss of granulosa cell identity and transdifferentiation of granulosa cells into Sertoli-like cells (Ottolenghi et al., 2005; Uhlenhaut et al., 2009).

Here we investigated the relationship between the early supporting cell lineage in the bipotential gonad and the postnatal granulosa cell population. Using the Foxl2tm1(GFP/cre/ERT2)Pzg (Foxl2GCE) mouse line to lineage-trace the fate of the early supporting cell precursors, we show that these cells specifically contribute to the population of follicles activated immediately after birth. In contrast, those primordial follicles that grow during adult life are comprised of granulosa cells specified long after sex determination, just prior to and throughout the postnatal follicle assembly period.

3.3 Results

3.3.1 FOXL2 marks a granulosa cell precursor lineage in the fetal ovary

In the mouse, FOXL2 protein expression is first apparent around E11.5 in a small number of somatic cells near the ovary/mesonephros boundary (Wilhelm et al., 2009). At later embryonic stages, FOXL2 is strongly expressed in the somatic cells lining germ cell nests, weakly expressed in the interstitial cells surrounding the ovarian vasculature, and excluded from the surface epithelium. In the early postnatal ovary, FOXL2 expression is apparent in both granulosa and interstitial cells, though it declines in the latter population with age (Cocquet et al., 2002; Schmidt et al., 2004; Guigon et al., 2005).
To determine whether FOXL2 specifically marks the descendants of the bipotential supporting cell precursors in the early mouse ovary, we immunostained XX Sry-EGFP transgenic gonads with a FOXL2 antibody and assessed colocalization at E12.5 and E13.5. Nearly all EGFP-positive cells colabeled with FOXL2 (Fig. 3A, B), indicating that XX cells capable of activating the Sry promoter upregulate Foxl2 after committing to the ovarian pathway. However, only a subset of the FOXL2-positive cells expressed EGFP, indicating possible heterogeneity in transgene expression or in the origins or differentiation status of FOXL2-positive cells.

We then extended our analysis forward in developmental time to determine whether this early population of Foxl2-expressing cells specifically gives rise to granulosa cells in the postnatal ovary or whether it contributes to multiple cell types. To lineage trace these cells, male mice expressing GFP-CreERT2 under the endogenous Foxl2 promoter (McMahon et al., 2008; Harding et al., 2011) were crossed to females carrying the R26R reporter (Soriano 1999), and pregnant females were injected with tamoxifen at E12.5. Embryos were allowed to develop to E14.5 or P14 before dissection.
Figure 3. FOXL2 is expressed by supporting cell precursors that are competent to express Sry. Ovaries from E12.5 (A) and E13.5 (B) XX Sry-EGFP transgenic embryos immunostained with antibodies against FOXL2 (green, nuclear) and EGFP (magenta, nuclear and cytoplasmic) show clear overlap (white) between the two markers (arrowheads in insets), though some FOXL2-positive cells were not EGFP-positive (arrows in insets). Confocal images were taken at a 200× magnification. Scale bars represent 20 μm. Modified from (Mork et al., 2011).
Figure 3: FOXL2 is expressed by supporting cell precursors that are competent to express Sry
The E14.5 XX Foxl2<sup>GCE</sup>; R26R samples confirmed the specificity of Cre activation in FOXL2-positive cells and also revealed that the GFP-CreERT2 fusion protein was expressed in most, but not all, cells labeled by the FOXL2 antibody at E14.5 (Fig. 4A, B). Ovaries from P14 Foxl2<sup>GCE</sup>; R26R animals were stained with an antibody against β-galactosidase to visualize the postnatal distribution of lineage-traced cells (Fig. 5). As predicted, the vast majority of cells expressing β-galactosidase were granulosa cells; a few β-galactosidase-positive cells could not be definitively identified and might have been stromal cells. Notably, β-galactosidase staining was not observed in the nascent theca cells surrounding each growing follicle (Fig. 5). Collectively, these results validate FOXL2 as a good marker of granulosa cell precursors in the embryonic ovary as well as the descendants of the bipotential supporting cell precursors.

Surprisingly, lineage-traced granulosa cells were only observed in the activated (growing) follicles in the center of the ovary, not in the primordial follicles concentrated in the cortex (Fig. 5A-D). Because our protocol targeted the first cells to express Foxl2, this result suggested a possible correlation between the stage at which a cell upregulates Foxl2 and the time at which its host follicle is activated. We hypothesized that this temporal relationship would be masked if Cre activation were delayed until later in development (e.g., E14.5). In this case, we expected that the much larger population of FOXL2-positive cells present at E14.5 would contribute to follicles in the cortex as well as the medulla. However, the distribution of lineage-traced cells in the ovaries of P9
Figure 4. Specificity and activation of the Foxl2-GCE transgene in the fetal ovary. (A) Ovaries from E14.5 transgenic Foxl2\textsuperscript{GCE/+}; R26R and control R26R embryos show that the GFP-CreERT2 fusion protein is specifically expressed in a subpopulation of FOXL2-expressing cells. (B) Ovary from a transgenic Foxl2\textsuperscript{GCE/+}; R26R embryo injected with tamoxifen at E12.5 and dissected at E14.5. β-galactosidase expression was specifically detected in a subset of FOXL2/GFP double-positive cells. Confocal images of whole-mount immunostained gonads were taken at 400×. Scale bars represent 20 μm. Modified from (Mork et al., 2011).
Figure 4: Specificity and activation of the Foxl2-GCE transgene in the fetal ovary
Figure 5. *Foxl2*-expressing cells in the fetal ovary give rise to granulosa cells in medullary follicles. (A-C) *Foxl2^{GCE+/-}; R26R* mice were exposed to tamoxifen at E12.5 and E14.5, dissected at P14 (A) or P9 (B, C), and stained with an antibody against β-galactosidase (magenta). Positive staining was nearly exclusive to granulosa cells in the large follicles in the medulla of the ovary. Arrow in (C) indicates a very rare lineage-traced granulosa cell in a primordial follicle. (D) High-magnification image of a P14 *Foxl2^{GCE+/-}; R26R* ovary from a mouse exposed to tamoxifen at E12.5, stained with antibodies against β-galactosidase (magenta) and FOXL2 (green). Lineage-labeled cells were observed in large secondary follicles as well as smaller primary follicles, but not primordial follicles. Arrowheads indicate growing follicles containing lineage-labeled cells that were sectioned through the edge rather than the middle of the follicle. Confocal images were taken at a magnification of 100× (A, B) or 400× (C, D). Nuclei (blue) were stained with syto13. Scale bars represent 50 μm. Modified from (Mork et al., 2011).
Figure 5: Foxl2-expressing cells in the fetal ovary give rise to granulosa cells in medullary follicles
mice exposed to tamoxifen at E14.5 (Fig. 5B, C) was nearly indistinguishable from those injected at E12.5 (Fig. 5A, D). Similar results were obtained with a higher dose of tamoxifen (data not shown). Therefore, the granulosa cells that populate the primordial follicles of the cortex must derive from cells that do not express the Foxl2GCE cassette at E12.5 or E14.5. Assuming that the Foxl2GCE cassette is activated in a random subset of cells expressing Foxl2 at E12.5-14.5, these results suggest that granulosa cells are produced in two waves: the first corresponding to the FOXL2-positive cells derived from bipotential supporting cell precursors, which furnish growing follicles of the medullary zone, and a second pool of unknown origin that contributes to primordial follicles assembled in the cortical region of the ovary and activated throughout adult life.

3.3.2 FOXL2-positive cells are arrested during embryonic development

A previous study of the proliferation dynamics of somatic cells in the perinatal rat ovary distinguished two populations of ovarian granulosa cells based on location (cortex vs. medulla) and proliferation history (Hirshfield 1992). Granulosa cells in cortical follicles were derived from cells that were actively proliferating during mid to late gestation, whereas those in medullary follicles derived from cells that had been quiescent (Hirshfield 1992; Hirshfield et al., 1995). Because our lineage-tracing results indicated that the medullary follicles of the postnatal mouse ovary derived from fetal Foxl2-expressing cells, we investigated whether these cells were quiescent during fetal life.
Previous microarray and immunohistochemical studies showed enrichment of Cip/Kip cyclin-dependent kinase inhibitors (p27, p21, and p57) in the somatic compartment of the embryonic ovary (Nef et al., 2005; Bouma et al., 2007; Cory et al., 2007; Rajareddy et al., 2007). To specifically assess the proliferation status of fetal FOXL2-positive cells, we colabeled wild-type E12.5 and E14.5 ovaries with FOXL2 and Ki67 (a marker of active cell cycle) or phospho-Histone H3 (pHH3; a marker of mitosis) (Fig. 6A, C and Fig. 7A, B). As hypothesized, all FOXL2-positive cells were Ki67- and pHH3-negative. Instead, they strongly expressed the cyclin-dependent kinase inhibitor p27 and appeared to have entered cell cycle arrest (Fig. 6B, D). Subsequent microarray analyses revealed that the levels of several cyclins (e.g., Ccna2, Ccnb2, Ccne2), cyclin-dependent kinases (e.g., Cdca2), and components of the cytokinesis complex (e.g., Cep55, Racgap1, Mklp1, Anln) are specifically depleted in this lineage relative to other cell types in male and female gonads (Jameson et al., unpublished), consistent with their persistent cell cycle arrest.

Mitotic arrest of the FOXL2-positive population persisted until after birth, when granulosa cells in activated medullary follicles resumed cycling and became Ki67-positive and p27-negative (Fig. 6E-H). Granulosa cells in primordial follicles remained arrested (Fig. 6H, inset).
Figure 6. FOXL2-positive cells are arrested throughout embryonic development. Cells expressing FOXL2 (green) were negative for Ki67 (magenta; A, C) and positive for p27 (magenta; B, D) at E12.5 and E14.5. At birth (E, F), the FOXL2-positive cells in close proximity to developing oocytes remained arrested, though cells more distant from oocytes were positive for Ki67 and negative for p27. Insets in (E) and (F) show FOXL2 expression in primordial follicles and surrounding interstitial cells, with or without Ki67 (E) or p27 (F) overlay. By P7 (G-H), a subset of follicles had progressed into primary and secondary stages. FOXL2-positive cells in these activated follicles were Ki67-positive (inset in G) and p27-negative, though granulosa cells in primordial follicles in the cortical region of the gonad remained arrested (inset in H). Oocytes upregulated p27 shortly after birth (F, H), as previously reported (Rajareddy et al., 2007). White color indicates overlap between the markers. (A-D) are images of whole-mount immunostained gonads taken at 400×. (E-H) are images of cryosectioned gonads taken at 200×. Scale bars represent 20 μm. Modified from (Mork et al., 2011).
Figure 6: FOXL2-positive cells are arrested throughout embryonic development
Figure 7. FOXL2-positive cells are not mitotically dividing, but the surface epithelium is actively cycling during early ovary development. Cells expressing FOXL2 (green) were negative for phospho-histone H3 (pHH3, magenta) staining at E12.5 (A) and E14.5 (B). At birth (C), pHH3 staining was generally excluded from strongly FOXL2-positive cells in the newly formed follicles. By P7 (D), a subset of follicles had progressed into primary and secondary stages and contained pHH3-positive cells (inset). Dividing cells were also observed in the stroma (arrowhead). (E-H) The surface epithelium contained Ki67-positive cells (magenta) at all stages examined. Nuclei (blue) were stained with syto13. (A, B, E, and F) are images of whole-mount immunostained ovaries taken at 400×. (C, D, G, and H) are images of cryosectioned gonads taken at 200× (C, D) or 400× (G, H). Scale bars represent 20 μm. Modified from (Mork et al., 2011).
Figure 7: FOXL2-positive cells are not mitotically dividing, but the surface epithelium is actively cycling during early ovary development
The second population of rat granulosa cells, which were incorporated into cortical primordial follicles, derived from cells that were actively dividing during fetal development (Hirshfield 1992). We determined that cells in the surface epithelium, which never express FOXL2 protein, and interstitial cells, which weakly express FOXL2 after E15.5, were actively cycling (Ki67-positive and p27-negative) at all stages examined (Fig. 7E-H and data not shown), marking them as possible sources of the second wave of granulosa cells.

3.3.3 During fetal life, new FOXL2-positive cells arise from cycling progenitors in the surface epithelium

A clear increase in the number of FOXL2-positive cells in the ovary was observed between E12.5 and E14.5 (Figs. 3A and 6C, D). Because FOXL2-positive cells are mitotically arrested, this increase must be attributed to recruitment rather than proliferation. This also means that the original supporting cell precursors specified during the bipotential period cannot account for the full population of FOXL2-positive cells present in the embryo. Previous work in the testis revealed that proliferation in the surface epithelium gives rise to Sertoli cell precursors during the bipotential period (Karl et al., 1998; Schmahl et al., 2000). By E11.5, the population of Sertoli cells that will form testis cords is fully allocated, and further divisions in the surface epithelium produce interstitial cells rather than new Sertoli cells (Karl et al., 1998). However, it remained possible that in the ovary, the surface epithelium (Sawyer et al., 2002) continues to contribute to the FOXL2-positive population throughout fetal development.
To test this idea, we labeled the surfaces of XX gonads with MitoTracker, a mitochondrial dye, and then cultured the samples for 2-48 h. In E11.5 gonads cultured for 2 h, the dye was clearly restricted to the surface epithelium of the gonad (Fig. 8A). After 6 h, the label was present in more cell layers, and after 48 h, labeled cells had ingressed deep into the ovary (Fig. 8B-D). To confirm that female supporting cell precursors derive from the surface epithelium, we labeled the surface of E11.5 Sry-EGFP ovaries with MitoTracker and cultured them for 48 h. Many EGFP-expressing cells in deeper layers of the gonad were labeled with MitoTracker, indicating that they were supporting cell precursors that had emerged from the surface epithelium of XX gonads after E11.5 (Fig. 8C). Consistent with this finding, in ovaries labeled at either E11.5 or E12.5 and cultured for 48 h, many ingressing cells expressed FOXL2 (Fig. 8D, E). Less inward movement and rare colocalization between MitoTracker and FOXL2 were observed in cultures labeled at E14.5 (Fig. 8F). These findings indicate that bipotential supporting cell precursors and embryonic FOXL2-positive cells do indeed derive from the surface epithelium but that this activity slows down after E12.5.
Figure 8. The surface epithelium is a source of new Sry-EGFP- and Foxl2-expressing cells in the fetal ovary. The ovarian surface was labeled with the cytoplasmic MitoTracker dye (MTO; magenta) at various stages of ovary development, and the samples were cultured for 2-72 h. (A) Gonads from E11.5 embryos fixed after 2 h show that the dye is confined to the outermost cell layers. (B) After 6 h, cell divisions in the coelomic epithelium generate labeled cells that move deeper inside the gonad. (C) Ovary from an Sry-EGFP transgenic embryo labeled with MitoTracker at E11.5. Many EGFP-positive cells contained the label after 48 h of culture (overlap is white; arrowheads and inset). (D) Similarly, in an E11.5 wild-type ovary cultured for 48 h, MitoTracker-labeled cells had ingressed to very deep layers, and many began to present nuclear FOXL2 staining (green; inset). (E, E') Gonads labeled at E12.5 displayed fewer ingressing cells that colabeled with FOXL2 after 24-48 h. (F, F') In samples labeled at E14.5, no ingression was observed after 24 h. Multiple cell layers were labeled after 48 h, but few ingressing cells were positive for FOXL2. Confocal images were taken at a magnification of 400× (A-E) or 200× (F). Scale bars represent 20 μm. Modified from (Mork et al., 2011).
Figure 8: The surface epithelium is a source of new \textit{Sry-EGFP-} and \textit{Foxl2}-expressing cells in the fetal ovary
Because the surface epithelium is proliferative and FOXL2-positive cells are arrested, cycling progenitors should enter mitotic arrest after exiting the surface epithelium but before upregulating Foxl2. To estimate the duration of this process, we performed a series of BrdU lineage tracing experiments (Fig. 9A). We confirmed that FOXL2-positive cells were not in S-phase (i.e., BrdU-negative) by exposing E12.5, E13.5, and E14.5 embryos to BrdU for 2 h and then staining their gonads with antibodies against FOXL2 and BrdU (Fig. 9A, B). In subsequent experiments, embryos were pulsed with BrdU at E11.5, E12.5, E13.5 or E14.5, injected with excess thymidine 2 h later, and then allowed to develop for 24-48 h before dissection. We then estimated the percentage of BrdU/FOXL2 double-positive cells within the total population of FOXL2-positive cells. At each stage, very few BrdU/FOXL2 double-positive cells were observed 24 h after injection. Conversely, in embryos pulsed at E11.5 or E12.5 and allowed to develop for 48 h, a significant increase in the proportion of double-positive cells was seen (p < 0.001; Fig. 9A, B), suggesting that there is typically a delay of more than 24 h between a progenitor cell’s last S-phase and upregulation of Foxl2. This spike was not observed in samples pulsed at E13.5 or E14.5 (Fig. 9B), suggesting that the number of new cells entering the population was proportionally much lower at these later stages. When embryos were pulsed at E15.5 or E16.5 and traced for 24-48 h, BrdU/FOXL2 colocalization was detected
Figure 9. Cycling progenitor cells give rise to new Foxl2-expressing cells. (A) Example of BrdU lineage tracing and data collection. In this series, pregnant females were injected with BrdU at E12.5, chased with thymidine 2 h later, and dissected after the indicated trace durations. Gonads were stained with antibodies against BrdU (magenta) and FOXL2 (green); white color indicates overlap between the markers. BrdU/FOXL2 double-positive cells (circles) were counted relative to the total number of FOXL2-positive cells and expressed as a percentage. Images were taken at a 400× magnification. The scale bar represents 20 μm. (B) Quantification of BrdU results. Embryonic stages listed above the bars indicate when BrdU was injected. n = 4-12 images per time point, from 2-4 independent gonad samples. Error bars indicate SEM. Embryos pulsed at E11.5 or E12.5 showed significantly higher proportions of BrdU/FOXL2 double-positive cells in 48-h traces relative to 24-h traces, but those injected at E13.5-14.5 did not, suggesting that the number of new cells entering the population was proportionally much lower at later stages. *p < 0.001; n/s, not significant. Modified from (Mork et al., 2011).
Figure 9: Cycling progenitor cells give rise to new Foxl2-expressing cells
in the weakly FOXL2-positive interstitial cells as well as a few other FOXL2-expressing cells near the surface epithelium, indicating that these two domains were still proliferative (data not shown).

3.3.4 New granulosa cells are generated in the ovarian cortex throughout the follicle assembly period

In the outbred mouse strain used for these experiments (CD1), follicle assembly begins at birth and is nearly complete by P5. At this time, almost all surviving oocytes are individually encapsulated within primordial follicles by a dedicated set of granulosa cells (Pepling et al., 2001). Many follicles in the medullary domain are activated and begin to grow immediately upon assembly (Hirshfield 1991), whereas cortical primordial follicles remain arrested (Fig. 6H). The mechanism by which some follicles are selected for activation remains unclear (Adhikari et al., 2009).

If the population of arrested FOXL2-positive cells specified before birth were sufficient to endow all surviving oocytes with a set of granulosa cells, no BrdU-positive granulosa cells should be detected in the primordial follicles of ovaries pulsed with BrdU during follicle assembly and traced until P7. Conversely, if a pool of cycling progenitors contributed to the granulosa cell population throughout this period, primordial follicles containing BrdU-positive granulosa cells should be easily detectable. To investigate these possibilities, we injected P1 and P4 female mice with BrdU and examined their ovaries at P7. Numerous BrdU-positive granulosa cells were detected in the primordial follicles of these samples (Fig. 10A, B), indicating that these cells were
still cycling when the BrdU was applied and thus did not derive from the arrested FOXL2-positive population. This finding is in agreement with the earlier studies of the rat ovary (Hirshfield 1992). We predicted that these cycling progenitors would reside in the surface epithelium, which was still proliferative at P1 (Fig. 10C, D). BrdU staining was also observed in small clusters of p27-negative, FOXL2-negative somatic cells residing just below the surface (Fig. 10C, D).

To determine whether new granulosa cells arise from the surface epithelium during the postnatal period, we labeled P1, P3, and P7 ovaries with MitoTracker and cultured the samples for 24-96 h. In all samples, the surface epithelium was very strongly labeled after 24-48 h, but almost no inward movement was observed (Fig. 11A and data not shown). When P1 and P3 ovaries were cultured for 72-96 h, the labeled domain clearly expanded to multiple cell layers. In contrast, in ovaries labeled at P7, when follicle assembly is nearly complete, labeled cells remained almost completely confined to the surface epithelium, even after 96 h of culture (Fig. 11A). Most ingressing cells did not express FOXL2 at these time points. However, we were able to detect several MitoTracker-labeled FOXL2-positive cells in primordial follicles (Fig. 11B) and in other less well-defined positions under the surface epithelium (Fig. 11A).
Figure 10. New granulosa cells arise in the ovarian cortex after birth. (A, B) Ovaries of P7 pups exposed to BrdU at P1 (A) or P4 (B) and stained with antibodies against BrdU (magenta) and α-laminin (green) show BrdU-positive granulosa cells inside primordial follicles (arrows, insets). As expected, granulosa cells in actively dividing medullary follicles were heavily labeled in samples pulsed at P4 but not in samples pulsed at P1. We speculate that the label was titrated out during the week-long chase in the latter case. (C, D) Somatic cells in and near the surface epithelium remain proliferative after birth. Ovaries from P1 mice were injected with BrdU 2 h prior to dissection and stained with antibodies against BrdU (magenta) and p27 (D) or FOXL2 (E) (green). Arrowheads point to clusters of proliferative (p27-negative, BrdU-positive) FOXL2-negative somatic cells under the surface epithelium. Nuclei (blue) were stained with syto13. Confocal images were taken at 200× (A, B) or 400× (C, D) magnification. Scale bars represent 20 μm. Modified from (Mork et al., 2011).
Figure 10: New granulosa cells arise in the ovarian cortex after birth
Figure 11. The ovarian surface epithelium gives rise to new granulosa cells during the follicle assembly period. (A) Ovaries from P1, P3 and P7 pups were labeled with the cytoplasmic MitoTracker dye (MTO, magenta), cultured for 24-96 h, then fixed and stained with an antibody against FOXL2 (green, nuclear). Only the surface epithelium was labeled after 24 h, but obvious ingression of labeled cells was observed after 72-96 h in cultures started at P1 or P3, but not P7. Arrowheads point to FOXL2-positive cells labeled with MitoTracker. (B) A few squamous MitoTracker-labeled FOXL2-positive cells (arrowheads) were incorporated into primordial follicles after 96 h in ovaries placed into culture at P3. Nuclei (blue) were stained with syto13. Confocal images were taken at a magnification of 200× (A) or 400× (B). Scale bars represent 20 μm. Modified from (Mork et al., 2011).
Figure 11: The ovarian surface epithelium gives rise to new granulosa cells during the follicle assembly period
To determine whether the FOXL2-negative ingressing cells were contributing to the stromal population, we used a transgenic line in which EYFP is expressed under control of the α-smooth muscle actin promoter (*Acta2-EYFP*). This reporter is generally excluded from follicles (Fig. 12A, B) but highly expressed in the stromal cells of the ovary, including many of the somatic cells proximal to the surface epithelium (Fig. 12B). To test the idea that many ingressing cells belong to the *Acta2-EYFP* population, we labeled the surface epithelium of P1 and P3 transgenic *Acta2-EYFP* ovaries and cultured them for 72-96 h. However, only a small number of ingressing MitoTracker-labeled cells expressed the EYFP reporter (Fig. 12C).

Therefore, cells ingressing from the surface epithelium of the perinatal ovary appear to contribute to three cell populations: FOXL2-positive granulosa cells, Acta2-EYFP-expressing stromal cells, and cells that express neither marker. However, given sufficient time, we expect that most ingressing cells would eventually express FOXL2 and/or *Acta2-EYFP* because the vast majority of non-endothelial somatic cells in the early postnatal ovary express one or both of these markers (see Fig. 6H and 12A). Longer cultures with MitoTracker were impractical, as the dye is progressively diluted at each cell division. Collectively, these results show that the proliferative ovarian surface epithelium generates granulosa cell precursors at two stages of ovary development, from at least E11.5-14.5 and during the postnatal follicle assembly period.
Figure 12. Interstitial cells also emerge from the surface epithelium after birth. (A) Ovary from a P2 Acta2-EYFP mouse showing strong reporter activity in the interstitial cells between newly formed follicles. Magenta, EYFP; green, FOXL2. (B) Some of the FOXL2-negative proliferative cells under the surface epithelium expressed the Acta2-EYFP reporter (magenta, arrowheads) at P1. (C) Ovaries from P1 Acta2-EYFP mice were labeled with MitoTracker (magenta) and cultured for 72 or 96 h. A subset of ingressing cells expressed the Acta2-EYFP reporter (green; arrowheads). The asterisk marks an oocyte that had incorporated MitoTracker. Nuclei (blue) were stained with syto13. Confocal images were taken at a magnification of 100× (A) or 400× (B, C). Scale bars represent 20 μm. Modified from (Mork et al., 2011).
Figure 12: Interstitial cells also emerge from the surface epithelium after birth
3.4 Discussion

The idea that supporting cell precursors in the murine bipotential gonad give rise either to adult Sertoli or adult granulosa cells is inaccurate, at least in its simplest form. Fetal FOXL2-positive cells derived from this population do not give rise to the granulosa cells present in the adult mouse. Instead, they specifically contribute to the subset of follicles in the medulla of the ovary that are activated immediately after birth and reach antral stages before puberty (Figs. 5, 13; (Adhikari et al., 2009)). Without the required stimulation from pituitary-derived FSH, the vast majority of these follicles are destined to be lost to atresia or converted into interstitial tissue (McGee et al., 1998; Adhikari et al., 2009). In contrast, the granulosa cells that equip the cortical primordial follicles and will be activated in adult life arise at more advanced stages, as late as the end of the postnatal follicle assembly period, a few days after birth (Figs. 10, 11, and 13).

These results may help to explain the findings of Ito et al. (Ito et al., 2006), who used a Sry-Cre transgenic line to trace the fate of female cells competent to activate the Sry promoter. Here, the authors detected positive reporter activity in the granulosa cells of a subset of follicles in prepubertal animals. It is unclear whether β-galactosidase activity was detected in primordial follicles as well as larger activated follicles. The results of the present work would suggest that this was not the case because Cre was only expressed during the earliest stages of gonad development (≤ E11.5) in female Sry-Cre animals (Ito
et al., 2006), so all such lineage-labeled cells would be expected to contribute to the activated medullary population.

Granulosa cells of dormant primordial follicles exist in stable cell cycle arrest for months, years, or even decades, waiting for the enigmatic activation signal that releases their proliferative potential and results in the final maturation of a Graafian follicle. Interestingly, the granulosa cell precursors specified early in development enter mitotic arrest concomitant with their embryonic specification, shortly before they upregulate Foxl2 (Figs. 6 and 9). Because they resume cycling upon follicle activation at birth (Fig. 6), their period of quiescence is relatively short. The cortical granulosa cells, in contrast, arise perinatally (~E15.5 to P4) from proliferating progenitors, enter mitotic arrest as they are assembled into follicles, and may remain arrested for the entirety of a female’s reproductive life.

While the granulosa cells of the medulla and cortex can be classified into two separate populations (Fig. 5; [27]), they are likely the descendants of a single progenitor source, born at different stages of development (Fig. 13). The surface epithelium of the gonad appears to be a major, if not the only, source of granulosa cell precursors, though other potential sources cannot be wholly discounted. The first cells to emerge from this epithelium (prior to E11.5) constitute the bipotential supporting cell precursor population, competent to activate the Sry promoter and differentiate as either granulosa or Sertoli cells (Fig. 8C; [6]). In the testis, the original Sry-expressing cells and their
Figure 13. Origins of ovarian granulosa cells. Divisions in the surface epithelium of the bipotential gonad (E11.5) produce both interstitial cells (beige) and supporting cell precursors (green) that differentiate as Sertoli cells in the presence of a Y-chromosome; in XX individuals, these cells move deep into the ovary and give rise to the granulosa cells in the medullary follicles activated immediately after their assembly at birth (green). As the ovary differentiates (~E14.5), further divisions in the surface epithelium contribute more cells to the granulosa population, though they remain closer to the ovarian surface. The granulosa cells that populate the primordial follicles in the cortex (pink), which are activated in adult life, arise from the surface epithelium around birth. By P7, follicle assembly is mostly complete, and surface cells no longer move into the ovary. Modified from (Mork et al., 2011).
Figure 13: Origins of ovarian granulosa cells
progeny account for all of the Sertoli cells present in the adult [8]: they assemble into testis cords by E12.5 and then accommodate the growing tubules by proliferating [33] rather than recruiting new cells into the population. This does not appear to be the case in the ovary because the number of FOXL2-positive cells increases in the absence of intrinsic proliferation (Figs. 6 and 9). New FOXL2-expressing granulosa cell precursors must therefore be recruited into the population after the bipotential period has concluded.

In accord with this observation, after sex is determined at E11.5, cells continue to drop out of the ovarian surface epithelium and, in some cases, enter mitotic arrest and upregulate Foxl2 (Figs. 8 and 9). Those that do not arrest likely contribute to the interstitial population (Fig. 12). Surprisingly, new granulosa cells were specified from cycling progenitors in the cortex as late as P4, toward the end of the follicle assembly period (Figs. 10 and 11). In contrast, no ingression from the surface epithelium was observed in ovaries labeled at P7, even after 96 h of culture, suggesting that the role of the ovarian surface epithelium as a source of new granulosa cells normally concludes coincident with the end of the follicle assembly phase. The signals that control the temporal activity of the coelomic epithelium at fetal and neonatal stages are unknown. However, cells of the ovarian surface epithelium retain the ability to move into the ovary in adult life, as this is an important part of the post-ovulatory repair process in the mature animal (Ahmed et al., 2007).
In addition to their distinct cellular origins, the first wave of follicles to be activated in the ovary exhibit several properties that distinguish them from follicles activated after puberty. They progress to antral stages almost twice as fast as adult follicles (Hirshfield 1991), present distinct morphological features (Hirshfield et al., 1995), and show altered expression of several steroidogenic enzymes and steroid receptors relative to similarly sized follicles in the adult (Galas et al., 2011). Differences between these two groups of follicles might be attributable to their different origins or to environmental factors such as gonadotropin levels and diet, which differ in prepubertal and adult animals. Such differences are consistent with the distinct functions of follicles activated before puberty, which establish ovarian functionality at the hormonal level prior to the first LH surge (Guigon et al., 2003), and those that grow during adult estrus cycles and are destined for ovulation/luteinization or atresia. In female rodents, which enter puberty around 30-40 days postpartum (Safranski et al., 1993; Ahima et al., 1997), the first oocytes to be ovulated may actually derive from the abnormal follicles activated at birth [40]. However, in larger mammals that enter puberty many months or years after birth, the prepubertal period is characterized by the development of multiple waves of anovulatory follicles (e.g., Adams et al., 1994; Mahdi et al., 2008); in some species, functionality of the hypothalamic-pituitary-gonadal axis is established early. FSH is produced, allowing such follicles to grow to antral stages, but the surge in luteinizing hormone required for ovulation is repressed by negative feedback from low levels of
estrogen until the animal is sufficiently mature to bear young (reviewed by Rawlings et al., 2003)).

The function and upstream regulators of the extended mitotic arrest of granulosa cell precursors remain uncertain. It is possible that mitotic arrest during the embryonic period prolongs and preserves the proliferative potential of granulosa cells for the time at which they will be tasked with the formidable demands of folliculogenesis. Alternatively, mitotic arrest may be required for the adoption of granulosa cell fate, or, more generally, supporting cell fate: preliminary work indicates that supporting cell precursors are arrested in both sexes during the bipotential period (data not shown). Mice carrying null mutations in the cyclin-dependent kinase inhibitors p27 or p21 do not have overt phenotypes in the embryonic ovary. However, female p27 single mutants have a larger endowment of primordial follicles at birth and exhibit premature ovarian failure and sterility after puberty, attributed to the failure of oocytes to remain meiotically arrested (Rajareddy et al., 2007). It is unknown whether embryonic granulosa cell precursors arrest normally in these mutants, in spite of the loss of the inhibitory factors, or whether their reentry into cycle supports the assembly of an increased number of primordial follicles.

Mutant models that fail to arrest or prematurely resume cycling may provide insight into these issues. While FOXL2 was primarily used as a marker in the present work, it has also been linked to disruptions in cell cycle. A somatic mutation in its forkhead
DNA-binding domain (C134W) is present in >95% of adult granulosa cell tumors (Shah et al., 2009; Jamieson et al., 2010), and its expression is markedly reduced in the juvenile form of this disease (Kalfa et al., 2007). In addition, a recent study showed that FOXL2 regulates the expression of several cell-cycle inhibitors and promotes G1 arrest in cultured granulosa cell lines (Benayoun et al., 2011). However, while FOXL2 may regulate the cell cycle in granulosa cells of the adult ovary, we do not attribute the embryonic mitotic arrest phenotype to the action of this protein because cell cycle arrest seems to precede FOXL2 expression (Figs. 9 and 15) and is established normally in mutant E14.5 Foxl2lacZ/lacZ (Schmidt et al., 2004) and hypomorphic E14.5 Foxl2GCE/GCE ovaries (pers. comm. from S. Jakob and R. Lovell-Badge; Fig. 19).

In conclusion, we show in the present work that the supporting cell precursors of the bipotential gonad specifically contribute to the rapidly depleted population of medullary follicles present in the prepubertal mouse. These precursors are mitotically arrested throughout fetal development but increase in number as new cells exit the surface epithelium and upregulate Foxl2. Granulosa cells of follicles activated during adult life arise from the ovarian surface epithelium perinatally. These findings add complexity to the standard model of sex determination in which the Sertoli and granulosa cells of the adult testis and ovary directly stem from the supporting cell precursors of the bipotential gonad.
4. Mitotic arrest of bipotential supporting cell precursors

4.1 Summary

The signals that instruct bipotential supporting cell precursors to adopt their distinct cell fate have not been elucidated. We determined that these precursors express markers of mitotic arrest toward the end of the bipotential period in both XX and XY gonads. Dividing cells in the coelomic domain thus give rise to progeny that enter mitotic arrest as they move out of the surface epithelium. Sertoli cells resumed cycling after sex determination, whereas female granulosa precursors remained arrested until postnatal stages, as described in section 3.3.2. We report the results of our investigation into candidate factors potentially responsible for inducing arrest during the bipotential period, maintaining it in female supporting cell precursors, and/or stimulating Sertoli cells to resume cycling. We determined that antagonism of Notch signaling may be required for precursor cells to adopt more differentiated fates, but ectopic activation of the pathway is not always incompatible with establishment or maintenance of cell cycle arrest. We also identified a role for Wnt signaling in maintaining cell cycle arrest in the ovary.

4.2 Introduction

In the mouse, the gonad forms around E10.0, a day and a half before sex is determined and the gonad begins to develop as a testis or ovary. Gene expression
differences between the sexes become apparent midway through this bipotential window, before any morphological differences can be detected (e.g., Nef et al., 2005; Beverdam et al., 2006). Many questions remain about this early phase of gonad development, including, for example, how precursor cells emerge from the coelomic epithelium and adopt their more differentiated cell fates. The mechanisms controlling the specification of the bipotential supporting cell precursors, herein defined as the cells competent to activate the Sry promoter (see section 3.3.1), is a particularly interesting problem that remains unresolved to date.

The bipotential mouse gonad (~E10.0-11.5) is positioned on the ventromedial surface of the mesonephros and consists of about four to six layers of pseudoepithelial somatic cells and a scattering of germ cells underneath a single-layered coelomic epithelium. Some, if not all, of the pseudoepithelial cells in the interior of the gonad stem from the coelomic epithelium rather than the neighboring mesonephric tissue (Karl et al., 1998). A low level of somatic proliferation, concentrated at or near the coelomic epithelium, was observed in both XX and XY gonads from E10.5 to E11.3 (Schmahl et al., 2000). Two pulses of increased XY-specific proliferation then occur in the coelomic domain: the first (E11.3-11.5) involves SF1-positive cells that contribute to both the Sertoli and interstitial populations, whereas the second (E11.5-12.2) involves SF1-negative cells that specifically generate interstitial cells (Schmahl et al., 2000). Throughout the late bipotential period (E11.3-11.5), little to no active proliferation was detected in the SF1-positive cells located
in the deeper layers of the gonad (Schmahl et al., 2000). Interestingly, experimental inhibition of proliferation from ~E10.8-11.2, but not before or afterwards, blocked testis cord development and expression of male markers (Schmahl et al., 2003), indicating that the initial sex-independent proliferation event, not the male-specific pulses, is critical for testis determination. This result has generally been interpreted to mean that male development requires that a threshold number of Sertoli progenitor cells be produced during the early proliferative period.

In the XY gonad, most of the pseudoepithelial cells upregulate Sry around E10.75 and commit to Sertoli cell fate (Bullejos et al., 2001); the remaining cells contribute to the interstitial population. Sry is expressed in XY gonads until ~E12.25 (Wilhelm et al., 2005); however, Sry expression can only activate the male program during a limited window spanning from ~E10.9 to E11.2 (Hiramatsu et al., 2009). This period overlaps almost perfectly with the critical window of proliferation mentioned above (Schmahl et al., 2003). These findings are summarized in Figure 14.

Interestingly, all of the known upstream regulators of Sry (e.g., Wt1, Gata4, and Map3K4) are expressed throughout the entire gonad, not just in the pseudoepithelial subset, suggesting that other factors are also required to make these cells ‘competent’ to express Sry and commit to supporting cell fate. Indeed, Hiramatsu et al. (2009) showed that even if expression of Sry is driven in all cells of the urogenital ridge, only the normal number of cells within the gonad express SOX9, supporting the idea that unknown
factors are required to establish Sertoli cell fate (Hiramatsu et al., 2009). In this section, we report a novel property of bipotential supporting cell precursors that may be involved in their cell fate specification.
Figure 14. Summary of key events during the bipotential period and early gonad differentiation. Different colors indicate the original study from which the data were obtained. The timeline of development is shown in terms of embryonic days and tail somite number.
Figure 14: Summary of key events during the bipotential period and early gonad differentiation
4.3 Results

4.3.1 Early mitotic arrest phenotype

To precisely determine the timing of cell cycle arrest relative to other events that occur during the bipotential stage of gonad development, we stained gonads from E10.5-12.5 XX and XY embryos with markers of active cell cycle (Ki67) or cell cycle arrest (cdk inhibitors p27 and p21). At E10.5, nearly all of the cells in the genital ridge were Ki67-positive in both sexes (Fig. 15A, B). However, from E11.0-11.5, many Ki67-negative somatic cells could be observed underneath the coelomic epithelium (Fig. 15C, D). This loss of Ki67 staining was accompanied by an upregulation of p27 and p21 in the same domain by E11.25 (Fig. 15I-L and data not shown).

The two sexes diverged after sex was determined at E11.5. By E11.75, Ki67 had been upregulated and p27 downregulated throughout XY gonads (Fig. 15F, N, P). Somatic p21 staining was last observed at E12.0 (Fig. 15R). To confirm that Sertoli cells had resumed cycling by E12.0, we performed a 1-h BrdU culture and then co-stained the gonads with SOX9. As expected, many SOX9-positive cells were BrdU-positive at this stage (Fig. 15H). Interestingly, cells resumed cycling in a center-to-pole pattern, such that p27-positive cells could still be detected at the anterior and especially the posterior poles of the gonad at E12.25 (Fig. 15S).

In XX gonads, a population of somatic cells remained in mitotic arrest (as judged by Ki67, p27, and BrdU staining (Fig. 15E, M, O, G, Q) until postnatal stages, as described
Figure 15. Mitotic arrest of bipotential supporting cell precursors. (A, B) Somatic cells are initially cycling in early XX (A) and XY (B) gonads. At E10.5, most GATA4-positive cells (blue) were Ki67-positive (green). (C, D) By E11.5, most GATA4-positive cells in the interior of the gonad were Ki67-negative, though those at the surface and germ cells and endothelial cells (stained with PECAM, magenta) remained Ki67-positive. (E, F) After sex determination, Ki67 staining in XX gonads (E) remained mostly restricted to the cells near the surface, germ cells, and endothelial cells, whereas many GATA4/Ki67-double positive cells were seen in the testis (F). (G, H) E12.0 XX and XY gonads were cultured in BrdU for one hour to label S-phase cells. In XX gonads (G), BrdU staining (magenta) was restricted to the surface domain and germ cells, and many p27-positive cells (green) were observed (similar to (O)). In XY gonads (H), BrdU frequently colocalized with SOX9 (green), indicating that Sertoli cells were actively cycling. (I-P) Inverse patterns were observed for p27 during this time period. No p27 staining (green) was detected in GATA4-positive somatic cells (blue) between E10.5 (not shown) and E11.0 (I, J), though strong expression was observed in both XX (K) and XY (L) gonads at E11.25. p27 levels had declined in the testis relative to the ovary by E11.75 (M, N) and almost disappeared from the center of the testis by E12.0 (O, P). (Q, R) p21 (green) was also detected in supporting cell precursors, but declined after E12.0. Germ cells were labeled with E-cadherin (blue). (S) Resumption of cell cycle in XY gonads occurred in a center-to-pole pattern, complementing the pattern of Sertoli cell differentiation (stained with AMH,
magenta). Germ cells and vasculature were labeled with PECAM (blue). A and P indicate the anterior and posterior ends of the testis, respectively. The dotted lines (A, B, Q, and R) indicate the gonad-mesonephros border. Scale bars represent 50 μm.
Figure 15: Mitotic arrest of bipotential supporting cell precursors
above (see section 3.3.2). p21, however, was downregulated in XX somatic cells after ~E12.5 (data not shown).

The patterns of mitotic arrest in XY and XX supporting cell precursors are depicted in Figure 14 relative to other important events that occur during the bipotential period and onset of gonadal differentiation.

**4.3.2 Candidate factors that initiate or maintain cell cycle arrest in supporting cell precursors**

We next attempted to identify the upstream pathways that regulate the establishment and maintenance of cell cycle arrest in supporting cell precursors. The Notch pathway and the transcription factor *Dmrt1* were recognized as candidate regulators for establishing mitotic arrest in this population because of the correlation between their expression domains and the observed patterns of proliferation and arrest within the gonad (see below). Germ cells also arrive in the gonad shortly before the onset of mitotic arrest, suggesting that they may provide a signal that induces somatic cells to stop dividing. In addition to these candidates, we examined several genes and pathways specific to the female pathway (e.g., *Foxl2*, canonical Wnt signaling) to determine whether they played a role in maintaining mitotic arrest in the granulosa cell precursor population.

**4.3.2.1 Active Notch signaling is not incompatible with cell cycle arrest**

The Notch antagonist NUMB is strongly and symmetrically expressed in pseudoepithelial somatic cells from E10.5 to E12.5 in XX and E10.5 to E11.75 in XY.
gonads (Fig. 16A, B). NUMB is also localized to the basolateral domain of cells in the coelomic epithelium (Fig. 16C, C'). Gonads from embryos expressing a transgenic Notch reporter (Duncan et al., 2005) show the inverse pattern, i.e., higher activity is generally seen in the coelomic epithelium (Fig. 16I). Of the four Notch receptors, Notch1 and Notch4 are specifically expressed in endothelial cells, Notch3 is upregulated in XY interstitial cells after sex determination, and Notch2 is broadly expressed throughout both male and female gonads at early stages (Fig. 16D, E and (Tang et al., 2008)). The latter is therefore best poised to transduce any signal between non-endothelial somatic cells early in development. Hes1, a downstream target of Notch signaling, is initially expressed in XY and XX interstitial cells (Fig. 16F, G) but also becomes upregulated in female somatic cells after E12.5 (Fig. 16H) coincident with the downregulation of NUMB (Fig. 16A).

We hypothesized that active Notch signaling in the coelomic epithelium drives cell division and may maintain these cells in an undifferentiated state. NUMB could then be asymmetrically distributed to pseudoepithelial daughter cells of the proliferating coelomic epithelium, and the resulting suppression of Notch might induce cell cycle arrest and/or commitment to supporting cell fate. Although the downstream effects of Notch signaling differ depending on cellular context, the pathway has been repeatedly implicated in the proliferation of many tumor cell types (Wang et al., 2009) as well as normal cells, e.g., T cells (Joshi et al., 2009), pituitary cells (Monahan et al., 2009), and
Figure 16. Notch pathway activation and antagonism in the early gonad. (A, D, F) Microarray data from four sorted cell populations (supporting cells (Sertoli and pregranulosa cells), interstitial/stromal cells, endothelial cells, germ cells) collected from XX and XY gonads at E11.5-13.5 (Jameson et al., unpublished). (A) The Notch antagonist Numb is enriched in the supporting cell lineage (blue lines) relative to other cell types at E11.5, then declines to background levels by E12.5 in XY gonads and E13.5 in XX gonads. (B) NUMB (magenta) is enriched in SOX9-positive pre-Sertoli cells (green) at E11.5. (C, C’) NUMB is also weakly expressed in the coelomic epithelium (stained with GATA4, blue) at E11.5 but appears to be asymmetrically distributed towards the basolateral edge of the cell. (D) Notch2 is highly expressed in male and female non-endothelial somatic cells at the early stages of gonad development. (E,E’) At E11.5, NOTCH2 (green) is expressed throughout the gonad (stained with GATA4, blue) and neighboring mesonephric tissue. (F) Hes1, a canonical downstream target of Notch signaling, is expressed in endothelial (red lines) and interstitial/stromal cells (purple lines) and becomes upregulated in female pregranulosa cells (blue dotted line) after E12.5, coincident with the downregulation of Numb. (G,H) At E13.5, HES1 protein (green) is enriched in XY interstitial and endothelial cells (G) and all female somatic cells except the coelomic epithelium (H), in accord with the microarray data. Endothelial and germ cells are labeled with PECAM (blue). (I, I’, I”) Gonads from E11.5 embryos expressing a transgenic Notch reporter (TNR, magenta). Reporter activity is enriched in
the coelomic domain and weak or absent in SOX9-expressing cells (green), the opposite of the NUMB localization pattern. TNR images courtesy of T. DeFalco. Scale bars represent 50 μm.
Figure 16: Notch pathway activation and antagonism in the early gonad
pseudoepithelial progenitor cells (Dong et al., 2010). HES1 also directly represses the expression of multiple cdk inhibitors, thus promoting progression through the cell cycle (Castella et al., 2000; Murata et al., 2005; Monahan et al., 2009). Therefore, NUMB-induced Notch antagonism may indirectly promote cell cycle arrest by downregulating Notch targets like Hes1 that inhibit arrest.

Because we hypothesized that the absence, not the presence, of Notch signaling was required to induce cell cycle arrest/supporting cell fate, we evaluated the effects of ectopic activation of the pathway. We took two complementary approaches to this problem. First, we induced constitutively active Notch signaling in gonadal somatic cells. Male Sfl-Cre or Wt1CreER (tamoxifen-inducible) mice were crossed to female mice with a floxed Notch1 intracellular domain (NICD) fragment fused to GFP in the Rosa26 locus (RosaNICD (Murtaugh et al., 2003)), and mutant embryos were assessed for colocalization between GFP and markers of cell cycle (p27 and Ki67) and supporting cell fate (FOXL2 and SOX9). Because the Sfl-Cre transgene is not expressed until ~E11.5 (Bingham et al., 2006), by which point cell cycle arrest has initiated, we used the Wt1CreER line to activate Notch signaling at early stages (E9.5-10.5). Neither of these approaches produced gonad-wide Cre activation, but the presence of the GFP marker allowed us to specifically evaluate cells that had undergone Cre-mediated recombination.

We frequently observed coexpression of GFP with p27 and FOXL2 in E12.5 XX Sfl-Cre; RosaNICD or Wt1CreER; RosaNICD mutant gonads (Fig. 17A). In XY mutants, SOX9
expression was detected in many GFP-expressing cells, as previously reported (Fig. 17B and (Tang et al., 2008)). These results indicate that active Notch signaling is not incompatible with cell cycle arrest or commitment to supporting cell fate, contrary to our hypothesis. However, we frequently observed small bulges of GFP-positive cells erupting from the coelomic surface of both male and female mutant gonads (Fig. 17C–E). A more detailed analysis of female mutants revealed that these cells were proliferative (Ki67-positive) (Fig. 17F), though occasionally a cell within a given cluster expressed either p27 or FOXL2 (Fig. 17G). This phenotype may indicate that activation of constitutive Notch signaling has different effects on a cell depending on whether it is in the coelomic epithelium or the deeper layers of the gonad when the Cre is activated. Cells that have already moved out of the epithelium may have already embarked on a differentiation trajectory that makes them resistant to ectopic Notch signaling. However, we hypothesize that constitutive Notch signaling affects the adhesive or proliferative properties of cells in the coelomic epithelium, preventing them from ingressing into the deeper layers of the gonad and adopting their normal fates. We expect that this phenotype would have been more pronounced if we had used a Cre that was more
Figure 17. Constitutive activation of Notch signaling is not incompatible with cell cycle or supporting cell fate. (A) Gonad from an E12.5 female Sf1-Cre; Rosa\textsuperscript{NICD} embryo immunostained with antibodies against p27 (magenta), FOXL2 (blue), and GFP (green). GFP marks the cells constitutively expressing the Notch1 intracellular domain (NICD). Co-localization between p27, FOXL2, and GFP was detected throughout the gonad, indicating that constitutive activation of the Notch pathway is not incompatible with cell cycle arrest of supporting cell fate. (B) Similarly, GFP colocalization with SOX9 was detected in a testis from an E13.5 Wt1-Cre\textsubscript{ER}; Rosa\textsuperscript{NICD} embryo, though co-expressing cells were somewhat rare. (C-E) However, numerous small bulges of GFP-positive cells were present on the surfaces of the mutant gonads (images taken at 100×, 200×, and 400× in C, D, and E, respectively). These bulges were mostly Ki67-positive (magenta in (F); inset shows GFP staining alone), though cells expressing p27 and FOXL2 were occasionally observed. The dotted line in D represents the ovary-mesonephros border. For the Wt1-Cre\textsubscript{ER}; Rosa\textsuperscript{NICD} cross, tamoxifen was orally administered to pregnant females at E9.5 and E10.5. Scale bars represent 20 μm.
Figure 17: Constitutive activation of Notch signaling is not incompatible with cell cycle or supporting cell fate
broadly expressed in the coelomic epithelium. Sf1, in particular, is not endogenously expressed in this domain in the XY gonad after E11.5 (Schmahl et al., 2000), which may explain why the bulges were more frequently observed in female samples.

In a complementary experiment, we assessed the roles of Numb in cell cycle arrest and the establishment of supporting cell fate. Homozygous Numb−/− embryos die at E11.5 from central nervous system defects (Zhong et al., 2000), precluding an analysis of potential gonadal defects. We determined that the brain defects were bypassed and viability was restored when we specifically deleted Numb at the time of gonad formation (~E10.0) using a ubiquitously expressed, tamoxifen-inducible CreER (ERCre). A Numb homolog, Numbl, is also expressed in the gonad, albeit weakly, at these early stages. Because Numbl mutant mice are viable, Cre-mediated deletion of Numb was performed on a Numbl mutant or heterozygous background. The presence of the R26R reporter allele on the ERCre; Numblox/flox; Numbl−/− background allowed us to identify the cells in which Cre had been activated.

β-galactosidase was widely expressed in mutant gonads upon exposure to tamoxifen (Fig. 18C, D, left panels). However, the rate of recombination was low in germ cells and supporting cell precursors relative to other somatic cell types (Fig. 18C, D, right panels). Future studies will determine whether (i) the ERCre is inefficiently activated in these cell types; (ii) mutant cells die; or (iii) mutant cells cannot adopt Sertoli or pregranulosa cell fate.
The coelomic surfaces of ERCre; Numbflox/flox; Numbl−/− mutant gonads were bumpy compared to controls, but no outpockets akin to those present in the RosaNICD mutants were observed (Fig. 18A, B). Instead, we observed large pockets of cells inside the mutant gonads (asterisks in Fig. 18E, F) that did not express characteristic markers of female supporting cells (FOXL2, p27), male supporting (SOX9, SF1), interstitial or Leydig cells (VCAM-1, SF1), germ cells (PECAM-1), or endothelial cells (PECAM-1) (Fig. 18C-F). However, these cells retained expression of LHX9 (Fig. 18G) and GATA4 (Fig. 18C) and were proliferative (p27-negative in females) (Fig. 18F).

LHX9 is considered a marker of undifferentiated cells in or derived from the coelomic epithelium (Birk et al., 2000; Mazaud et al., 2002). In XY gonads, this marker is downregulated upon commitment to Sertoli cell fate but maintained in a subset of interstitial cells thought to represent Leydig cell precursors or other undifferentiated progenitors (Mazaud et al., 2002; DeFalco et al., 2011). In XX gonads, LHX9 expression is maintained in the supporting and interstitial lineages for a longer duration, consistent with their less robust differentiation program (DeFalco et al., 2011). GATA4 is expressed in somatic cells of the early gonad (except the endothelial cells) but absent from the neighboring mesonephros. Retention of LHX9 and GATA4 and loss of p27 expression in these mutant pockets, in the absence of other markers of differentiated cell types, thus indicates that these cells represent undifferentiated, proliferative, gonadal somatic cells.
Figure 18. Loss of Numb/Numbl disrupts gonad structure and differentiation. Bright field images show that XY (A) and XX (B) ER<sup>Cre</sup>; Numb<sup>fl<sub>ox</sub>/fl<sub>ox</sub></sup>; Numbl<sup>−/−</sup>; R26R mutants are morphologically abnormal with irregular cord structures (A) and bumpy coelomic surfaces. (C, D) β-galactosidase expression (magenta) from the R26R reporter suggests that ER<sup>Cre</sup> was extensively activated by our tamoxifen administration protocol. No β-galactosidase expression was observed in embryos lacking the ER<sup>Cre</sup> transgene (not shown). However, few β-galactosidase-positive germ cells (stained with PECAM; blue), Sertoli cells (strongly stained by GATA4; green), and FOXL2-expressing cells (green) were observed in mutant gonads. Right panels in (C) and (D) are higher magnifications of the boxed areas in the left panels and illustrate the general paucity of β-galactosidase staining inside XY testis cords (outlined in right panel of (C)) and in XX FOXL2-expressing cells. (E, F) Large clusters of cells within the mutant gonads did not express markers of any differentiated cell type. (E) VCAM1 (green) is expressed by all non-endothelial cells in the interstitium of the control testis (left panel), SF1 (magenta) is expressed by Leydig and Sertoli cells, and PECAM (blue) is expressed by endothelial cells and germ cells. Several large patches of VCAM1-negative, SF1-negative, and PECAM-negative cells (asterisks) were detected within the mutant testis (right panel). (F) Similar patches were observed in female mutants immunostained for p27 (magenta), FOXL2 (green), and PECAM (blue). The absence of p27 staining indicates that these cells are not mitotically arrested. (G) The undifferentiated patches (arrow) did express LHX9
(magenta; boxed area in middle panel is magnified in the right panel) and GATA4 (see panel (C)). Control gonads were \textit{Numb}^{\text{flox/flox}}; \textit{R26R} and wild-type, heterozygous, or homozygous for the \textit{Numbl} mutation. The genotype of all mutants was \textit{ERCre}; \textit{Numb}^{\text{flox/flox}}; \textit{Numbl}^{-/-}; \textit{R26R}. Tamoxifen was orally administered to pregnant females at E9.5 and E10.5. Scale bars represent 50 \text{\mu m}.
Figure 18: Loss of Numb/Numbl disrupts gonad structure and differentiation
These experiments therefore suggest that antagonism of Notch signaling by NUMB/NUMBL is involved in cell differentiation and initiation of cell cycle arrest. Future studies will need to determine the degree to which the absence of Numb in a given cell prevents it from adopting a more differentiated fate and whether this depends on the cell’s differentiation status when Cre recombinase activity is induced. Indeed, β-galactosidase expression was observed in many cells that appeared to be differentiated in addition to the pockets of undifferentiated cells. We also plan to explore the mechanisms underlying the defect in the organization of the coelomic surface by performing live imaging analyses or close inspection of the distribution of mutant and wild-type cells in this domain. Earlier administration of tamoxifen would likely induce a more severe phenotype in the Numb mutants, but we have evidence suggesting that this may also induce early lethality.

4.3.2.2 The presence of germ cells is not required to induce cell cycle arrest

Supporting cell precursors enter mitotic arrest around the same time that germ cells begin to proliferate in the gonad. We investigated the possibility that germ cells are required to initiate or maintain mitotic arrest in somatic cells by treating E10.5 embryos with busulfan, a chemotherapeutic drug that depletes primordial germ cells (Merchant 1975). However, FOXL2-positive cells remained in cell cycle arrest at E14.5 in treated embryos (Fig. 19A-D), indicating that the presence of germ cells is not required to establish or maintain arrest at this stage.
Figure 19. Neither germ cell depletion nor mutations in *Dmrt1*, *Foxl2*, or *Wnt4* affected the establishment of mitotic arrest in FOXL2-positive cells. FOXL2-positive cells (green) in XX E14.5 control gonads were Ki67-negative (A, magenta) and p27-positive (B, magenta). (C, D) Germ cell depletion resulted in compaction of FOXL2-positive cells but not re-entry into cycle. Asterisks mark remnant germ cells (blue) that were not depleted by the busulfan treatment. (E, F) FOXL2-expressing cells remained Ki67-negative (E) and p27-positive (F) in XX E14.5 *Dmrt1*-/- gonads. (G, H) In E14.5 *Foxl2*<sup>GCE/GCE</sup> hypomorphs, no differences in the distribution of Ki67 (G) or p27 (H) staining were observed relative to wild-type controls. (I, J) In XX E14.5 *Wnt4*<sup>+/+</sup> gonads, FOXL2-positive cells present both above and below the ectopic coelomic vessel (dotted line) were Ki67-negative and p27-positive. Ki67 and p27 staining patterns of littermate controls for *Dmrt1*, *Foxl2*<sup>GCE/GCE</sup>, and *Wnt4* mutants all resembled those of wild-type samples (A, B). Confocal images of whole-mount immunostained gonads taken at 400×. Scale bars represent 20 μm.
Figure 19: Neither germ cell depletion nor mutations in Dmrt1, Foxl2, or Wnt4 affected the establishment of mitotic arrest in FOXL2-positive cells.
4.3.2.3 *Dmrt1* does not regulate mitotic arrest in the fetal ovary

*Dmrt1* is a conserved gene that is involved in multiple aspects of gonad development, including the activation of *Stra8* expression in female germ cells (Krentz et al., 2011), pluripotency and proliferation in male germ cells (Krentz et al., 2009), and the maintenance of testis fate at postnatal stages (Matson et al., 2011). DMRT1 is expressed in germ cells and a subset of somatic cells in both XX and XY gonads at early stages, and turns off in XX somatic cells after E12.5 (Fig. 20A-D, Lei et al., 2007). DMRT1 shows an unusual expression pattern in the bipotential gonad in that it is excluded from the coelomic epithelium but expressed in the cells immediately underneath it as well as smattering of pseudoepithelial cells in the deeper layers (Fig. 20A). This pattern suggests that *Dmrt1* is upregulated immediately after cells exit the coelomic epithelium, which we hypothesize to also be the time at which they enter mitotic arrest. We therefore examined whether mitotic arrest was disrupted in female *Dmrt1*−/− mutants at E14.5. No differences were observed in p27 or Ki67 staining in the mutants relative to littermate controls (Fig. 19E, F), indicating that DMRT1 is not required for the establishment or maintenance of cell cycle arrest.
Figure 20. DMRT1 expression in the bipotential gonad and early stages of ovary development. (A) At E11.5, DMRT1 (green) is excluded from the coelomic epithelium but expressed in the somatic and germ cells immediately underneath it as well as smattering of mesenchymal cells in the deeper layers. GATA4 (blue) labels all somatic cells in the gonad, and PECAM (magenta) is expressed by endothelial and germ cells (B). At E12.5, DMRT1 closely colocalizes with FOXL2 (blue) in the somatic population and is also expressed in germ cells. Note that it is no longer expressed directly underneath the coelomic epithelium. (C-D) DMRT1 is downregulated in most somatic cells by E13.5 (arrowhead points to rare DMRT1-positive somatic cell) and is exclusively expressed in germ cells at E14.5. (A’-D’) show DMRT1 staining only. The scale bar represents 50 μm.
Figure 20: DMRT1 expression in the bipotential gonad and early stages of ovary development
4.3.2.4 *Foxl2* does not regulate mitotic arrest in the fetal ovary

We next assessed somatic mitotic arrest in *Foxl2* mutant embryos. Previous work showed that granulosa cell proliferation was impaired in postnatal *Foxl2*−/− ovaries relative to wild-type littermates, though in this case, the defect appeared to be a failure to resume cell cycle upon follicular activation rather than a failure to arrest. We therefore expected that somatic cell cycle arrest would proceed normally in embryonic *Foxl2*<sup>GCE/GCE</sup> mutants, a hypothesis borne out by the normal patterns of Ki67 and p27 staining observed at E14.5 (Fig. 19G, H). This analysis thus refuted the possible involvement of this gene in establishing or maintaining cell cycle arrest in the embryonic ovary. However, several studies have recently reported that most human granulosa cell tumors carry a Cys134Trp mutation in the *FOXL2* gene, implying that the protein may act to repress granulosa cell proliferation in the adult.

4.3.2.5 Wnt signaling is required to maintain, but not initiate, mitotic arrest

To investigate whether Wnt signaling, a critical pathway involved in female fate determination, regulates mitotic arrest of female somatic cells, we assessed the proliferation status of FOXL2-positive cells in *Wnt4*<sup>−/−</sup> mutant gonads. Despite the dysmorphic appearance and the presence of an ectopic coelomic vessel in the mutant ovaries, FOXL2-positive cells were arrested normally at E14.5 (Fig. 19I, J). However, after ~E15.5, downregulation of p27 was observed in the FOXL2-expressing cells at the anterior end of the gonad (Maatouk et al., unpublished data), coincident with the loss of
germ cells in this domain (see section 8.2.3). These patterns indicate that Wnt4 is unlikely to be the primary signal driving arrest in the early gonad but appears to be required for its maintenance at later stages of ovary development.

We next assessed whether the mitotic arrest phenotype was affected by constitutive activation of canonical Wnt signaling. In: Sf1-Cre; β-catenin\(^{\alpha 3/3}\) gonads, a stabilized form of β-catenin that cannot be targeted for destruction by GSK3- is expressed upon activation of Cre around E11.5 in Sf1-expressing gonadal somatic cells. XY Sf1-Cre; β-catenin\(^{\alpha 3/3}\) embryos undergo male-to-female sex reversal (Maatouk et al., 2008), underlining the critical involvement of Wnt signaling in ovary development. XX transgenic ovaries were larger and more convex than wild-type ovaries, suggesting that activated β-catenin might induce changes in proliferation or cell shape. Indeed, FOXL2-positive cells initially arrested normally in these mutants, but then resumed cycle around E14.5 (Fig. 21A-D). Excessive proliferation of granulosa precursors likely contributes to the hyperplastic and disorganized phenotype of the postnatal Sf1-Cre; β-catenin\(^{\alpha 3/3}\) mutant ovaries (Fig. 21E, F). This phenotype concurs with that reported by Boerboom et al. (2005), who showed that stabilization of β-catenin in granulosa cells after E17.5 often results in tumor formation (Boerboom et al., 2005).
Figure 21. Stabilization of β-catenin causes female somatic cells to prematurely resume cycle. (A) At E14.5, controls show strong p27 staining in FOXL2-positive cells. (B) Sf1-Cre; β-catenin<sup>lox/lox</sup> mutant littermates show dramatically reduced p27 expression. (C, D) This phenotype became even more obvious at E15.5, where many Ki67/FOXL2 double-positive cells could be detected in mutants (inset). (E, F) Premature resumption of cycle likely contributes to the disorganized morphology of postnatal mutant samples, where Ki67 staining shows that cells across the entire ovary were actively cycling. (A, B) are images of whole-mount immunostained gonads taken at a magnification of 400×; C-F are images of cryosectioned gonads taken at 200× (C, D) or 8× (E, F). Scale bars represent 20 μm.
Figure 21: Stabilization of β-catenin causes female somatic cells to prematurely resume cycle
4.3.3 Candidate factors that prompt Sertoli cells to resume cycling

Sertoli cells resume cycling shortly after sex determination in a center-to-pole pattern that parallels the formation of testis cords (Fig. 15S). Sertoli cells at the anterior pole, however, lose p27 expression prior to those at the posterior pole, which are commonly still arrested at E12.5 (not shown). Subsequent growth of the nascent testis cords occurs through proliferation of Sertoli cells and germ cells, as no new cells move into the cords after their initial formation. Numerous pathways involved in Sertoli cell differentiation and testicular morphogenesis are known to be active between E11.5 and E12.5. We therefore tested whether any of the following candidates were likely to be involved in stimulating Sertoli cell proliferation.

4.3.3.1 Migrating endothelial cells do not appear to stimulate Sertoli cell proliferation

One of the earliest morphogenetic events that occurs in the developing mouse testis involves the migration of endothelial cells into the gonad from the vascular plexus in the neighboring mesonephros (Martineau et al., 1997). These cells appear to migrate along tracks that define the future interstitial space between testis cords, then coalesce into a large vessel at the coelomic surface while maintaining the lateral branches between cords (Cool et al., 2008; Combes et al., 2009). When endothelial migration is blocked, a pronounced defect in cord formation occurs (Tilmann et al., 1999; Combes et al., 2009). This defect is largely attributable to a failure of the nascent interstitium to proliferate and drive wedges between the newly formed testis cords (Cool et al., 2011). We tested
whether the invading vasculature also provides signals that promote Sertoli cell proliferation by blocking endothelial migration and assessing p27 expression. In one set of experiments, we treated cultured gonads with su5402, a pharmacological inhibitor of tyrosine kinase receptors that can inhibit the VEGF receptor (Mohammadi et al., 1997) and is toxic to endothelial cells in this tissue. XY samples treated with this inhibitor for 24 h, beginning around E11.5, showed disrupted cord structures and no coelomic vessel (Fig. 22A, B). Greater cord disruption was observed in samples that were younger at the beginning of the culture period. In one set of samples, strong p27 staining colocalized with AMH (a marker of Sertoli cells), indicating that the treatment had prevented these cells from reentering cell cycle. However, no increase in p27 staining was seen in subsequent experiments (Fig. 22A, B), so this result remains unexplained.

Nevertheless, this experiment was flawed because su5402 may affect multiple important signaling pathways in the gonad (e.g., FGF) and the results cannot be specifically attributed to a loss of endothelial cells. We therefore eliminated the vasculature in the testis using a different approach involving selective inhibition of the VEGF pathway, which we previously showed to be critical for endothelial migration (Cool et al., 2011). For this experiment, embryos were exposed to VEGF trap (Regeneron), a drug that binds to VEGF ligands and prevents them from activating their cognate receptors. The drug was delivered via intraventricular injection following (Cool et al., 2011), and gonads were cultured for 24 h before fixation. This treatment effectively
blocked the formation of a coelomic vessel in XY samples. No consistent differences in p27 levels were observed (Fig. 22C, D), however, consistent with the majority of the su5402 experiments. Collectively, these experiments show that it is unlikely that migrating endothelial cells stimulate Sertoli cells to resume active cell cycle.

4.3.3.2 Fgf9 is unlikely to regulate the resumption of cell cycle in Sertoli cells

Another candidate factor that may promote Sertoli cell reentry into cell cycle is FGF9. This growth factor is expressed in the bipotential gonads of both sexes, but becomes significantly upregulated in XY supporting cell precursors around the time of sex determination. FGF9 is required to maintain Sox9 expression in the testis; consequently, mutations in Fgf9 lead to male-to-female sex reversal in mice. XY Fgf9−/− mutant gonads also show reduced proliferation at the early bipotential stage (E11.2) and during the subsequent male-specific pulse in SF1-positive precursors (Schmahl et al., 2004). FGF9 is also thought to promote the assembly of testis cords in a center-to-pole manner (Hiramatsu et al., 2010). We hypothesized that if FGF9 were responsible for inducing Sertoli cells to resume cycling, ectopic application of FGF9 to XX gonads might cause female somatic cells to exit mitotic arrest. Accordingly, XX gonads were explanted at E11.5 and cultured next to beads coated with FGF9 or in medium containing FGF9. Though this treatment induced an ectopic coelomic vessel and weak upregulation of SOX9, as previously reported (Kim et al., 2006), no loss of cell cycle arrest markers was observed (Fig. 22E, F). We also examined XY Fgf9−/− gonads to determine whether the
sex-reversing supporting cell precursors remained arrested or resumed cycling. Resumption of cell cycle would indicate that neither FGF9 nor SOX9 likely regulates this process. However, strong p27 staining was observed in E12.5 XY $F_{gf9}^{-/-}$ gonads, similar to control females (Fig. 22G-I). We believe that it is more prudent to interpret this result as further evidence of general sex reversal rather than a sign that FGF9 in particular is required for reentry into cell cycle.

4.3.3.3 Inhibition of retinoic acid signaling does not affect Sertoli cell cycle

Another event that occurs shortly after sex determination in XY gonads is upregulation of $C_{yp26b1}$ in Sertoli cells. This protein functions to metabolize retinoic acid (RA). Initiation of meiosis in female gonads appears to be stimulated by an influx of RA from the mesonephros (Bowles et al., 2006; Trautmann et al., 2008; Griswold et al., 2011). CYP26B1 is thought to metabolize RA in XY gonads, thereby reducing local levels of the signal and protect male germ cells from the meiosis-initiating signal (Bowles et al., 2006). Indeed, loss of $C_{yp26b1}$ in XY gonads causes male germ cells to prematurely enter meiosis, and exposure of XY gonads to supraphysiological levels of RA in culture induces upregulation of meiosis-related genes (Bowles et al., 2006). We therefore tested whether Sertoli cell proliferation was related to suppression of RA signaling by treating XY gonads with RA in culture. We observed upregulation of a meiosis-related gene, STRA8 (Fig. 22J, K), but no persistence of cell cycle arrest in these samples (Fig. 22L, M).
Figure 22. Candidate signals that prompt Sertoli cells to resume cycling. (A-D) Migrating endothelial do not appear to stimulate Sertoli cells to resume cycling. Endothelial cells were depleted in E11.5 XY gonads using two pharmacological inhibitors, su5402 (A, B) and VEGF trap (C, D). PECAM staining (green) shows the distribution of endothelial and germ cells in control and treated gonads. Note the absence of a coelomic vessel in treated samples. No increase in p27 staining (magenta) was seen with either treatment relative to controls. (E-I) FGF9 probably does not regulate mitotic arrest in supporting cell precursors. XX gonads cultured next to beads coated with BSA (E) or FGF9 (F) (circles) showed a small, local increase in SOX9 expression (green, arrows) but no reduction in p27 staining (magenta). (G-I) In contrast to male Fgf9<sup>+/−</sup> controls (H), supporting cells remain arrested (p27-positive, magenta) in XY Fgf9<sup>−/−</sup> sex-reversed gonads (I), comparable to female littermate controls (G). This result is consistent with either a requirement for FGF9 in stimulating cell cycle in Sertoli cells or a general consequence of sex reversal. PECAM staining (green) shows the distribution of endothelial and germ cells in control and mutant gonads. (J-M) Inhibition of retinoic acid signaling is unlikely to regulate Sertoli cell re-entry into cycle. (J, K) Addition of retinoic acid to the culture medium overwhelms causes XY germ cells (magenta) to ectopically express STRA8 (green). (L, M) However, this treatment does not prevent Sertoli cells from re-entering cell cycle, as p27 staining (magenta) remained at control levels. Scale bars represent 20 μm.
Hypothesis 1: Migrating endothelial cells induce Sertoli cells to resume cycling

![Image A: XY CONTROL](image1.png) ![Image B: XY CONTROL + su5402](image2.png) ![Image C: XY CONTROL + VEGF trap](image3.png)

Hypothesis 2: FGF9 induces Sertoli cells to resume cycling

![Image E: XX CONTROL](image4.png) ![Image F: XX CONTROL + FGF9 bead](image5.png) ![Image G: CONTROL XX Fgf9^+/+](image6.png) ![Image H: CONTROL XY Fgf9^+/+](image7.png) ![Image I: MUTANT XY Fgf9^-/-](image8.png)

Hypothesis 3: Inhibition of retinoic acid signaling causes Sertoli cells to resume cycling

![Image J: XY CONTROL](image9.png) ![Image K: XY CONTROL + RA](image10.png) ![Image L: XY CONTROL](image11.png) ![Image M: XY CONTROL + RA](image12.png)

Figure 22: Candidate signals that prompt Sertoli cells to resume cycling

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4.4 Discussion

This analysis identified mitotic arrest as a novel property of bipotential supporting cell precursors. This mitotic arrest is transient in XY embryos, as Sertoli cells resume cycling shortly after the end of the bipotential window at E11.5. In XX embryos, the descendants of the bipotential supporting cell precursors remain arrested throughout the rest of embryonic development and resume cycling upon postnatal follicle activation (see section 3.3.2). The functional significance of mitotic arrest in the specification of supporting cell precursors during the bipotential window remains unclear. Because the onset of Sry expression appears to precede the exit of these cells from cell cycle, mitotic arrest cannot be a prerequisite for Sry upregulation, but it may still be involved in the commitment of these cells to supporting cell fate.

In this analysis, we assessed whether cell cycle arrest could be normally established and maintained in the presence of elevated Notch signaling (Figs. 17 and 18) or mutations in Dmrt1 (Fig. 19E, F) or Foxl2 (Fig. 19G,H), in the absence of germ cells (Fig. 19C, D), or in mutants with gain- or loss-of function mutations in the Wnt pathway (Fig. 19I, J and Fig. 21). None of these manipulations completely prevented the establishment of cell cycle arrest. Activation of the Notch pathway (RosaNICD or Numb/Numbl mutants) was technically not incompatible with cell cycle arrest or establishment of supporting cell fate, but preliminary analyses suggest that many mutant cells remain in a non-arrested, undifferentiated state (Figs. 17 and 18). Neither germ cell depletion nor
mutations in Dmrt1 or Foxl2 caused female granulosa cell precursors to prematurely exit cell cycle arrest (Fig. 19C-H). However, a subset of cells at the anterior end of the ovary did resume cycling around E14.5 in XX Wnt4−/− embryos (Maatouk et al., unpublished data), and FOXL2-positive cells also began to divide prematurely in mutants expressing a stabilized form of β-catenin (Fig. 21). It is surprising that both loss and gain of Wnt signaling caused FOXL2-positive cells to precociously resume cycling. Despite this similarity, the phenotypes of the two mutants are quite distinct, and we suspect that resumption of cell cycle may have different implications in each condition. Because of the lack of an early loss of function phenotype in the Wnt4 mutant gonads, we conclude that the Wnt pathway is likely involved in the maintenance rather than the establishment of somatic mitotic arrest in the early gonad.

Because this candidate approach has not proved particularly effective at identifying factors that regulate mitotic arrest in the gonad, future studies will instead use pharmacological and viral strategies to block cell cycle arrest or ectopically force normally dividing cells to arrest. We plan to block cell cycle arrest using an adeno-associated virus (AAV) to infect the coelomic epithelium and deliver T121. T121 is an SV40 T antigen that inactivates the Rb family of proteins and induces progression of cells through the cell cycle (Pan et al., 1998). Using this approach, we will determine whether cells driven through the restriction point of the cell cycle, R, show reduced differentiation. We anticipate that cells expressing T121 will not arrest in G0/G1.
However, it is possible that although T121 will block arrest in G0, because p53 is unaffected, many cells may arrest at a later stage of the cycle (i.e., prior to synthesis or prior to mitosis). If so, it will be important to determine whether cells arrested at later stages of the cycle can establish expression of markers of supporting cell fate. We predict that arrest prior to S-phase is required based on previous literature (Levitan et al., 1998; Ambros 1999).

We also plan to induce cell cycle arrest in all cells of the gonad at E11.0-11.5 to determine whether ectopically arrested cells (i.e., cells in the coelomic epithelium or at the gonad/mesonephric border) activate Sry in an expanded domain and/or adopt supporting cell fate. E11.0 gonads will be explanted to organ culture and treated with pharmacological inhibitors that preferentially inhibit CDKs 4 and 6 (e.g., fascaplysin and CINK4), to block the progression of the cell cycle at G0/G1, or block CDKs 1 and 2 (e.g., roscovitine and purvalanols), to preferentially block the cell cycle at M and S (Shapiro et al., 1999). Expansion of the domain of cells that can be induced to differentiate as supporting cells simply by arresting their cell cycle would suggest (i) that cell cycle arrest is a critical competence factor for adoption of supporting cell fate, and (ii) that the number of cells in arrest is a mechanism that limits the number of cells susceptible to signals that commit cells to a particular cell fate, as previously suggested (Ambros 1999).

In the present study, we also evaluated several candidate factors related to the male pathway that might stimulate Sertoli cells to resume cycling at the end of the bipotential
window. Experimental manipulations of migrating endothelial cells, Fgf9, and retinoic acid signaling did not yield strong evidence in favor of any of these candidates (Fig. 22), though the results were in some cases inconsistent, and the assays are not necessarily definitive. As resumption of cycle may be a cell-autonomous consequence of the upregulation of the male pathway by Sry/Sox9, it may be difficult to identify the precise signals regulating this event.

It is interesting, nonetheless, that Sertoli and granulosa cell precursors are mitotically arrested from their earliest specification through their assembly into the functional units of the testis and ovary, i.e., testis cords and ovarian follicles, and only resume cycling when their host testis cord or follicle begins to expand. This phenomenon complements our observation that Sertoli and granulosa cell precursors emerge from the coelomic epithelium during a window spanning the earliest stages of gonad formation through the beginning of testis cord formation or the end of follicle assembly, after which the influx of cells slows or ceases entirely (Figs. 8 and 11, (Karl et al., 1998)). These parallels become even more intriguing when we consider that testis cord formation occurs long before follicle assembly. We therefore postulate that i) the resumption of cell cycle in growing testis cords might be stimulated by similar mechanisms that rouse dormant primordial follicles in the adult ovary; and ii) the signals that determine when precursor cells should stop ingressing from the coelomic epithelium may be similar in the two sexes.
5. Molecular identification of cell lineages in the developing turtle gonad

5.1 Summary

We characterized the cellular composition of the red-eared slider turtle gonad during and after the bipotential period by examining the distribution of proteins known to be involved in mammalian sex determination and gonadogenesis (WT1, GATA4, LHX9, SOX9, and CYP19A1) as well as a marker highly expressed by endothelial cells (CAV1). We also analyzed the distributions of α-laminin, collagen, and several components of adherens and tight junctions to further our understanding of medullary cord structure. The three somatic populations, i.e., the coelomic epithelium, medullary cord cells, and interstitial cells, can be distinguished by their respective locations within the gonad as well as the complement of transcription factors they express. Endothelial cells were not detected until later stages of testis differentiation.

5.2 Introduction

The surprising diversity of sex-determining mechanisms observed in vertebrates does not extend to a comparable diversity in the structure and function of the gonads. Indeed, the gonads of adult vertebrates are quite similar, with the exception of modifications in the ovary that accommodate eggs of different sizes and yolk content. Ultrastructural similarities are also present at fetal stages, though variation has been observed in the migration route of germ cells and their initial position within the gonad.
(e.g., Fujimoto et al., 1976; Pieau et al., 1999; Molyneaux et al., 2004), the presence or absence of primitive cord structures in both presumptive sexes (e.g., Wibbels et al., 1991; Renfree et al., 1996), the length of the bipotential period, and contribution of cells from the mesonephros (e.g., Martineau et al., 1997; Sekido et al., 2007; Diaz-Hernandez et al., 2008; Carmona et al., 2009). However, many of the transcription factors and signaling pathways (other than Sry itself) that regulate mammalian testis and ovary development have proved to be evolutionarily conserved and transcribed in the gonads of all vertebrates tested (e.g., Morrish et al., 2002; Loffler et al., 2003; Yao et al., 2005; Shoemaker et al., 2007; Shoemaker et al., 2007), though functional conservation cannot be easily confirmed in non-genetic model systems.

In the present study, we characterized the cellular composition of the red-eared slider turtle gonad during and after the bipotential period by examining the distribution of proteins known to be critical for mouse sex determination and gonadogenesis. This analysis provided a basis for subsequent studies on the origins of certain lineages and the involvement of estrogen and other signaling pathways in turtle gonad development (see section 6). Below, we briefly review the cellular composition of the early mouse gonad as well as previous work on cellular mechanisms of turtle gonad development, both of which served as foundations for the present analysis.
5.2.1 Cellular composition of the early mouse gonad

In mice, the gonad forms as a thickening of the coelomic epithelium on the ventromedial surface of the mesonephros around embryonic day (E)10. Following a brief bipotential period, sex is determined in the gonad by E11.5, and morphological differentiation of the testis is evident shortly thereafter. During the bipotential period, five different populations of cells are present within the gonad. The most distinct cell types are the germ cells, which migrate to the gonad from their distant site of specification, and the endothelial cells, which form a rudimentary vasculature that is elaborated later in development, more notably in the testis.

The other three somatic cell types, the supporting cell precursors, the interstitial/stromal progenitors, and the coelomic epithelium, show somewhat overlapping expression patterns during early development but can be distinguished by their locations within the gonad. The supporting cell precursors are the first cell type to adopt a sex-specific fate; in XY embryos, these cells express Sry, the male sex-determining gene, commit to Sertoli cell fate, and then direct the subsequent development of the testis (reviewed by Kashimada et al., 2010). In XX embryos, this population gives rise to a specific subset of granulosa cells in the postnatal ovary (see section 3.3.1). The interstitial cells of the testis are a somewhat mixed population of cells that proliferate in the space between the nascent testis cords and help to define their shape (Cool et al., 2011). A subset of this interstitial population differentiates into
steroidogenic Leydig cells, whereas others form the peritubular myoid cells that coat the
testis cords, and still others become closely associated with the blood vessels that form
between the cords. In the early ovary, the small population of interstitial cells remains
somewhat undifferentiated and closely associated with the ovarian vasculature
(Maatouk et al., unpublished data). The coelomic epithelium is the third population of
somatic cells in the early gonad. These cells appear to be relatively undifferentiated and
proliferative (see sections 3.3.2 and 4.3.1). During early development, their progeny
move into the deeper layers of the gonad and differentiate as interstitial or supporting
cell progenitors ((Karl et al., 1998); see section 3.3.3). In the testis, cells stop moving in
from the coelomic epithelium by E12.5 (Karl et al., 1998), but this process continues until
postnatal life in the ovary (see section 3.3.4).

Several transcription factors have been implicated in the earliest events of murine
gonadogenesis, including, among others, Wilms’ tumor 1 (Wt1), LIM homeobox gene 9
(Lhx9), and GATA binding protein 4 (Gata4). These proteins are expressed in the somatic
cells of the bipotential gonad before sex-specific differentiation begins (Pelletier et al.,
1991; Armstrong et al., 1993; Viger et al., 1998; Birk et al., 2000; Albrecht et al., 2001;
Mazaud et al., 2002; Tevossian et al., 2002; Jorgensen et al., 2005) and continue to function
in later testis and ovary development. As both WT1 and LHX9 promote proliferation of
the early somatic cell population, mutations in either gene lead to gonadal agenesis
(Kreidberg et al., 1993; Birk et al., 2000), while hypomorphic mutations in Gata4 severely
affect both testis and ovary differentiation (Tevosian et al., 2002; Manuylov et al., 2008). A fourth gene, sex-determining region Y box 9 (Sox9), has been associated with testis development in all vertebrates examined to date (Morrish et al., 2002) and is directly activated by the mammalian sex-determining gene SRY in mouse Sertoli cell precursors (Sekido et al., 2008). Sox9 is weakly expressed in both XX and XY mouse gonads during the brief bipotential period, but is quickly downregulated in the developing ovary (reviewed in Kobayashi et al., 2005).

5.2.2 Early gonad development in the red-eared slider turtle

In the red-eared slider turtle Trachemys scripta, the bipotential, temperature-sensitive period (TSP) spans from the initial appearance of the gonads around stage 14 to stage 19 at the female-producing temperature (FPT) and stage 20 at the male-producing temperature (MPT) (Wibbels et al., 1991; Ramsey et al., 2007), corresponding to 10 days at the FPT and 20 days at the cooler MPT. Previous histological analyses revealed the presence of primitive sex cords in the medullary domain of both presumptive sexes (Wibbels et al., 1991; Wibbels et al., 1993), distinguished from the surrounding interstitial cells on the basis of their epithelial characteristics, including the close apposition of the cord cell nuclei as well as a basement membrane lining the exterior of each cord. The coelomic epithelium, where the germ cells initially reside and from which the ovarian cortex will develop, is separated from the medullary compartment by another layer of basement membrane (Wibbels et al., 1991). This latter structure is contiguous with the
basement membrane lining the cords, in accord with early cell tracing studies showing that the medullary cords likely derive from invaginations of the coelomic epithelium (Yao et al., 2004).

Signs of testicular and ovarian differentiation are evident by stage 21 and pronounced by stage 26 (hatching). If the gonad commits to the ovarian fate, the cords disaggregate and rearrange to form circular lacunae (Pieau et al., 1999), while germ cells and somatic cells proliferate in the cortex (Schmahl et al., 2003) prior to the onset of meiosis and the organization of follicle-like structures. If eggs are incubated at the MPT, the medullary cord cells differentiate as Sertoli cells, engulf germ cells, and form definitive testis cords as the interstitial space between the cords expands and the coelomic epithelium contracts into a simple surface epithelium (Wibbels et al., 1991).

Below, we extend these previous studies of turtle gonad development to the molecular level by examining the distribution of proteins known to be critical for mouse sex determination and gonadogenesis

5.3 Results

5.3.1 Medullary cord structure

During early ovarian differentiation (stages 19 and 21), the basement membrane (revealed by \( \alpha \)-laminin staining) that delineates the cortical and medullary compartments thickens, while the deeper layers fragment as the medullary cords reform into small lacuna structures (Fig. 23A; (Wibbels et al., 1991)). These circular
arrangements are also lined by basement membrane at the hatching stage (26) (Fig. 23A). At the MPT, between stages 19 and 21, the testis cords expand and mature to assume a more tubular configuration as the germ cells move into the cords from their previous position at the coelomic surface. The basement membrane surrounding the cords becomes more evenly distributed and organized during this process (Fig. 23B). By the hatching stage, most of the cords have pinched off from the surface epithelium and are segregated from the interstitium by this layer of extracellular matrix (Fig. 23B). Similar patterns were observed with an antibody against type I collagen (Fig. 24C).

To further investigate how medullary cord structure is maintained, we used antibodies against β-catenin, E-cadherin and ZO-1 to determine whether adherens or tight junctions were present. At the early stages of gonad development, β-catenin strongly outlines germ cells as well as the somatic cells of the coelomic epithelium and medullary cords (Fig. 24A, B). The interstitium was labeled very weakly or not at all. Later, in the differentiating ovary (Fig. 24A, right panel), medullary expression of β-catenin diminished as the cords broke apart, whereas the cortex remained brightly labeled. As cord structures matured in the developing testis, β-catenin was again observed to outline the somatic and germ cells inside the cords as well as the remaining layer of surface epithelium (Fig. 24B, right panel), in a pattern reminiscent of the E15.5 mouse testis (Chang et al., 2008). Although the expression of this protein was not sex-specific, the fact that it was downregulated in the ovarian medulla relative to the cortex
Figure 23. Cord structures are defined by α–laminin deposition during turtle gonadogenesis. (A) At the FPT, α–laminin (green) outlines medullary cord structures throughout the bipotential stages (15-19), but begins to fragment after the TSP (≥ 21). The basement membrane that separates the cortical and medullary compartments (arrowhead) begins to thicken by stage 21. The medullary cords reform into small circular lacuna structures lined by α–laminin (arrow) by stage 26. (B) At the MPT, the α-laminin deposition pattern resembles that of FPT gonads at early stages but becomes progressively smoother and more organized throughout testis cord morphogenesis. By stage 26, most of the cords have pinched off from the surface epithelium and are separated from the interstitium by basement membrane. Nuclei were stained with syto13 (blue). Germ cells present large, dim nuclei. The vertical grey line separates the bipotential stages from later sexual differentiation. FPT stage 26 picture courtesy of L. DiNapoli. The scale bar represents 20 μm.
Figure 23: Cord structures are defined by α-laminin deposition during turtle gonadogenesis
Figure 24. Medullary cord cells express markers of adherens and tight junctions. (A, B, left and middle panels) Midway through the TSP (stages 17 and 19), strong β-catenin staining was observed in the plasma membranes of germ cells as well as the somatic cells of the coelomic epithelium and medullary cords (A, B). The interstitium was labeled very weakly or not at all. In the hatching stage ovary (A, right panel), medullary expression of β-catenin diminished as the cords broke apart, whereas the cortex remained brightly labeled. The dashed line represents the border between the cortex and medulla. In the hatching stage testis (B, right panel), β-catenin staining remained strong in the Sertoli and germ cells inside the testis cords (outlined by dotted line) as well as the tunica albuginea at the surface. In MPT stage 19 gonads, the distribution of type I collagen (C) resembled that of α-laminin, whereas E-cadherin (D) and ZO-1 (E) resembled β-catenin. Images of whole-mount immunostained gonads were taken at 400×. Scale bars represent 20 μm.
Figure 24: Medullary cord cells express markers of adherens and tight junctions
makes it a useful marker of ovarian differentiation. The E-cadherin and ZO-1 expression patterns resembled that of β-catenin at the stages examined (Fig. 24D, E). These patterns suggest that adherens and tight junctions may help to maintain cord structure in the developing turtle gonad.

### 5.3.2 Characterization of molecular markers

Given the significant differences in the structure and organization of turtle and mouse gonads, we investigated whether WT1, GATA4, LHX9, and SOX9 showed parallel cell type-specific expression patterns.

#### 5.3.3.1 WT1 and GATA4 are expressed in all somatic lineages of the developing gonad

In the mouse, WT1 protein is expressed in all somatic cells of the early bipotential gonad, and gradually becomes restricted to Sertoli cells and the coelomic epithelium in the maturing XY gonad (Table 2; (Pelletier et al., 1991; Armstrong et al., 1993; Albrecht et al., 2001)). GATA4 is also initially expressed in all somatic cells, but by E13.5 it becomes upregulated in Sertoli cells relative to interstitial cells in XY gonads and slightly downregulated until post-natal stages in developing ovaries (Table 2; (Viger et al., 1998; Albrecht et al., 2001; Tevosian et al., 2002; Jorgensen et al., 2005)). A preliminary analysis of WT1 protein distribution in the turtle gonad was previously reported (Schmahl et al., 2003); the more detailed expression pattern presented here was used to inform the analysis of the other three genes. During the initial stages of turtle gonadogenesis, WT1 and GATA4 were expressed in the nuclei of all somatic lineages, including the cells of
the coelomic epithelium, the medullary cord cells, and the interstitial cells (Figs. 25 and 26; Table 2). In the differentiating ovary (FPT stages 19–26), WT1 expression was maintained in the rearranging medullary cords and the cortical somatic cells, while expression was absent in the interstitial population by the hatching stage (Fig. 25A, (Schmahl et al., 2003)). GATA4 was maintained in all somatic lineages (Fig. 26A). At the MPT (stages 19–26), the strongest WT1 expression was found in the pre-Sertoli cells of the developing testis cords, as in the mouse. Lower expression was detected in the surface epithelium and the interstitium until close to the hatching stage, when the only positive cells lay within the cords (Fig. 25B, (Schmahl et al., 2003)). In contrast, GATA4 expression typically declined in Sertoli cells relative to the interstitium and the surface epithelium, but some samples showed equivalent expression levels between cell types (Fig. 26B). This pattern is the reverse of that seen in the mouse testis.

5.3.3.2 LHX9 expression is restricted to the coelomic epithelium and interstitium

In mouse and chick gonads, LHX9 is initially expressed in all somatic cells, but quickly turns off in differentiating Sertoli cell precursors of the XY gonad such that the distribution of LHX9 and Sertoli markers becomes complementary (Table 2; (Birk et al., 2000; Mazaud et al., 2002)). This pattern was strikingly replicated in the early turtle gonad. At both temperatures, cells in the coelomic epithelium robustly expressed LHX9, while a subset of interstitial cells also showed weak staining (Fig. 27). No staining was observed in the medullary cord cells, where SOX9 was highly expressed (see below).
Figure 25. **WT1 is strongly expressed throughout turtle gonadogenesis.** (A) At the FPT, WT1 (red) is expressed in all somatic lineages throughout the bipotential stages, but declines in the interstitial population after the TSP (note the WT1-negative cells in the medulla of the stage 21-26 ovaries). The dashed line in the stage 26 panel represents the border between the cortex and medulla. (B) At the MPT, WT1 is initially expressed in all somatic lineages but becomes restricted to the coelomic epithelium and Sertoli cells during testis differentiation. Nuclei were stained with syto13 (blue). Images of whole-mount immunostained gonads were taken at 400×. The vertical grey line separates the bipotential stages from later sexual differentiation. The scale bar represents 20 μm. Modified from (Barske et al., 2010).
Figure 25: WT1 is strongly expressed throughout turtle gonadogenesis
Figure 26. GATA4 marks all somatic cell types during gonad development. (A) At the FPT, GATA4 (yellow) is expressed in all three somatic cell types throughout gonadogenesis. The dashed line in the stage 26 panel represents the border between the cortex and medulla. (B) At the MPT, GATA4 is initially expressed in all somatic lineages but declines in Sertoli cells in later stages. Nuclei were stained with syto13 (blue). Images of whole-mount immunostained gonads were taken at 400×. The vertical grey line separates the bipotential stages from later sexual differentiation. The scale bar represents 20 μm. Modified from (Barske et al., 2010).
Figure 26: GATA4 marks all somatic cell types during gonad development
Figure 27. LHX9 expression is restricted to the coelomic domain during turtle gonadogenesis. (A) At the FPT, LHX9 (red) is expressed strongly in the coelomic epithelium and very weakly in the interstitium during the bipotential stages. After sex determination, LHX9 marks the proliferating somatic cells of the coelomic epithelium (stage 21) as well as the small cortical cells arranged in primitive follicle structures around germ cells (stage 26). The dashed line in the stage 26 panel represents the border between the cortex and medulla. (B) At the MPT, LHX9 was also highly expressed in the coelomic epithelium of the bipotential gonad, but staining was frequently cytoplasmic in the single-layered surface epithelium of the differentiating testis (stage 26). Germ cell nucleoli were also strongly labeled by this antibody, but we believe this is an artifact, as we have seen similar effects with other antibodies that are also not expected to label germ cells. Nuclei were stained with syto13 (blue). Images of whole-mount immunostained gonads were taken at 400×. The vertical grey line separates the bipotential stages from later sexual differentiation. The scale bar represents 20 μm. Modified from (Barske et al., 2010).
Figure 27: LHX9 expression is restricted to the coelomic domain during turtle gonadogenesis
After the onset of ovarian differentiation (FPT stage 21), the somatic cells of the cortex increased in number and continued to strongly express LHX9. In a mature hatching stage ovary (26), small LHX9-positive somatic cells were present in nascent follicular structures around germ cells in the cortex. The surface epithelium that coats the outer edge of the cortex was also LHX9-positive, but staining was generally excluded from the nucleus (Fig. 27A). In the differentiating testis, LHX9 expression became restricted to the surface epithelium and showed progressively less nuclear localization (Fig. 27B).

5.3.3.3 SOX9 expression is lost from medullary cord cells at the FPT after sex determination but is maintained in the pre-Sertoli cells of the testis

In the mouse, SOX9 protein is expressed in Sertoli cell nuclei in the XY gonad and excluded from the XY coelomic epithelium and interstitium. SOX9 protein has been detected at low levels in the cytoplasm of XX somatic cells prior to sex determination, but is quickly downregulated thereafter (Table 2; reviewed in Kobayashi et al., 2005)). Similar to mouse, turtle SOX9 expression was very rarely seen in cells of the coelomic epithelium and never in the interstitium at either temperature throughout gonadogenesis. Instead, SOX9 expression was restricted to the medullary cord cells from the earliest stages (Fig. 28A, B). At the FPT, SOX9 expression peaked around stage 17 and then declined at stage 19 as the cords began to reform into lacunae. The timing of cord dissolution was variable, as some stage 19 gonads contained intact medullary cords, whereas others exhibited obvious lacuna morphology by this stage.
Downregulation of SOX9 expression is correlated with this morphological event (Moreno-Mendoza et al., 1999; Torres-Maldonado et al., 2001). By stage 21, SOX9 was completely absent from the developing ovary except for a few cells along the mesonephric border (Fig. 28A).

At the MPT, SOX9 expression was also very strong during the bipotential stages. As the cords became more defined and tubular (stages 21–26), the pre-Sertoli cells lining the inside of the testis cords maintained strong SOX9 expression (Fig. 28B). This association of SOX9 with the male pathway once testis fate is determined mirrors the pattern seen in other vertebrates (Morrish et al., 2002). However, the presence of strong SOX9 expression in both presumptive sexes throughout the bipotential period is not a consistently observed trait (e.g., Oreal et al., 1998; Nakamoto et al., 2005; Jorgensen et al., 2008).

### 5.3.3 SOX9 downregulation precedes cortical proliferation

Reorganization of cord structures in the medullary domain of the ovary (stage 18-20) generally precedes the onset of germ cell proliferation in the cortex (~19-20) (Wibbels et al., 1991), but the interdependence and relative timing of SOX9 downregulation and the pulse of somatic proliferation have not been previously investigated. It is possible that successful SOX9 downregulation requires a signal from the cortex produced by the proliferating somatic cells. To compare the timing of these two events, we briefly
**Figure 28. SOX9 expression becomes male-specific after sex determination.** (A) At the FPT, SOX9 (yellow) is initially expressed in medullary cord cells, but declines dramatically after sex determination. The dashed line in the stage 26 panel represents the border between the cortex and medulla. (B) At the MPT, SOX9 is highly expressed in medullary cord cells/Sertoli cells at all stages examined. Nuclei were stained with syto13 (blue). Images of whole-mount immunostained gonads were taken at 400×. The vertical grey line separates the bipotential stages from later sexual differentiation. The scale bar represents 20 μm. Modified from (Barske et al., 2010).
Figure 28: SOX9 expression becomes male-specific after sex determination
exposed gonads from stage 18-22 FPT and MPT embryos to BrdU and then immunostained them for BrdU and SOX9. SOX9 expression was present in all FPT samples at stage 18, very weak or absent in 5/8 and 6/8 gonads at stages 19 and 20, respectively, and absent in all stage 21 and 22 ovaries (Fig. 29A). Strong SOX9 expression was detected in MPT samples at all stages examined (data not shown). Enrichment of proliferating somatic cells in the cortical domain did not occur until stage 21 in FPT gonads, though many dividing germ cells were observed by stage 20 (arrowheads in Fig. 29A; (Wibbels et al., 1991)). These data indicate that SOX9 is downregulated before proliferation increases in the cortex of developing ovaries, in line with histological data on medullary cord regression (Wibbels et al., 1991).

To confirm that proliferation is not required for the establishment of female fate as measured by the absence of SOX9 staining, we cultured stage 18 FPT gonads with aphidicolin for six days and then assessed SOX9 expression. As predicted by the time course data, SOX9 expression was downregulated normally in the absence of proliferation (Fig. 29B).
Figure 29. SOX9 downregulation occurs prior to and independently of the burst of proliferation in the cortical domain during turtle ovary development. (A) Enrichment of proliferating somatic cells in the cortex was not observed until stage 21, whereas SOX9 downregulation occurred by stage 19-20. Proliferating cells were identified by BrdU (magenta) incorporation. The proportion of gonads with substantial nuclear SOX9 staining (green) is noted in each panel. Dashed lines indicate the border between the cortex and medulla. Arrowheads point to BrdU-positive germ cells. (B) Inhibition of proliferation did not impede SOX9 downregulation in differentiating ovaries. Stage 18 FPT gonads were cultured with aphidicolin for six days, briefly exposed to BrdU to confirm that proliferation was blocked in treated samples, and then immunostained for BrdU (magenta) and SOX9 (green). No nuclear SOX9 staining was observed in treated or control ovaries. Nuclei were stained with syto13 (blue). Images of cultured gonads were taken at 40× (A) or 200× (B). Scale bars represent 20 μm.
Figure 29: SOX9 downregulation occurs prior to and independently of the burst of proliferation in the cortical domain during turtle ovary development
5.3.4 Male-specific vascular development is delayed in the turtle relative to the mouse

At the hatching stage, the turtle testis is heavily vascularized, especially in comparison to the ovary, which lacks an obvious vascular network (Fig. 30A, B; (Crews et al., 1991)). No coelomic vessel could be detected at the surface of the testis (Fig. 30A), in contrast to the developing mouse testis, where the appearance of the coelomic vessel represents an important early male-specific morphological event that occurs shortly after sex determination.

To further investigate the development of the vasculature in the turtle gonad, we used an antibody against CAV1 that specifically labels endothelial cells in the early mouse gonad (Bullejos et al., 2002). CAV1-positive cells were very rarely observed in turtle gonads between stages 15 and 21 but were occasionally present at the gonad-mesonephros border (data not shown). However, in stage 26 MPT embryos, CAV1 staining was prominent in the cytoplasm of a subset of endothelial-like cells in the interstitium between the testis cords, linked in a manner suggestive of small blood vessels (Fig. 30C). Positive staining was also present at this stage in cells near the surface epithelium, though again, no contiguous blood-filled coelomic vessel was evident (Fig. 30C). In the stage 26 ovary, weak positive staining was observed in the coelomic epithelium in cells that did not exhibit endothelial morphology (Fig. 30D). Interestingly, in the mouse, CAV1 is expressed by female supporting cell precursors as well as
endothelial cells (Jameson et al., unpublished), suggesting a potentially conserved additional role for this protein in ovary development.

In the mouse, the endothelial cells that form the testis-specific vasculature actively migrate into the gonad from the neighboring mesonephros shortly after sex determination (Martineau et al., 1997; Cool et al., 2008). No such migration was observed in the early ovary. It is possible that the endothelial cells in the differentiating turtle testis enter into the tissue via a similar wave of migration from the mesonephros; however, previous attempts to evaluate this possibility were inconclusive (Yao et al., 2004).
Figure 30. Vascularization of the turtle testis. At hatching, the turtle testis (A) is heavily vascularized relative to the ovary (B). No coelomic vessel could be detected at the surface of the testis. (C) CAV1-positive endothelial-like cells (green) were distributed in the interstitium between the testis cords, forming what appeared to be small vessels. Positive staining was also present at this stage in cells near the surface epithelium. (D) In the stage 26 ovary, weak CAV1 staining was observed in the coelomic epithelium in cells that did not exhibit endothelial morphology. The dashed line represents the border between the cortex and medulla. Nuclei were stained with syto13 (blue). Images of whole-mount immunostained gonads were taken at 200×. tc, testis cord. Scale bars represent 50 μm.
Figure 30: Vascularization of the turtle testis
5.4 Discussion

We extended early histological studies describing the cell types present in the fetal turtle gonad during and after the bipotential period by examining the distribution of proteins known to be critical for mouse sex determination and gonadogenesis (WT1, LHX9, GATA4, and SOX9). This analysis was intended to complement the many recent studies that have examined the expression of conserved gonadogenesis genes by RNA-level approaches that could not identify cell-specific patterns (Ramsey et al., 2007; Rhen et al., 2007; Shoemaker et al., 2007; Shoemaker et al., 2007; Smith et al., 2008; Valenzuela 2010). We show that the three main somatic lineages of the turtle gonad (coelomic epithelium, medullary cords, interstitium) are defined by their combinatorial transcription factor expression profiles well before the end of the bipotential period when sex is finally determined. The cell type-specific expression patterns of the bipotential gonad and later testis/ovary development are largely conserved between the mouse and turtle, in spite of significant differences in gonad structure and morphogenesis (Wibbels et al., 1991; Yao et al., 2004). The most notable instances of conservation were i) the expression of WT1 and GATA4 in all somatic cell types of the early bipotential gonad; ii) the complementary relationship between LHX9 in the coelomic epithelium/interstitium and SOX9 in Sertoli precursor cells; iii) the progressive restriction of WT1 expression to Sertoli cells in the testis; and iv) expression of WT1, GATA4, and LHX9 in the cells thought to be granulosa precursors (Table 2). The
Table 2: Summary of cell type-specific expression patterns in the mouse and turtle

<table>
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<th>Mouse bipotential gonad (E11.5)</th>
<th>Ovary (E13.5)</th>
<th>Testis (E13.5)</th>
<th>Refs.</th>
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<td>++</td>
<td>+</td>
<td></td>
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<td>++ +/-</td>
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<td>LHX9</td>
<td>++ +</td>
<td>++</td>
<td>+</td>
<td>3, 4, 5, 6</td>
</tr>
<tr>
<td>SOX9</td>
<td>- +</td>
<td>-</td>
<td>-</td>
<td>7, 8, 9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Turtle bipotential gonad (stage 15)</th>
<th>Ovary (stage 26)</th>
<th>Testis (stage 26)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
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<td>++ +/-</td>
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<td></td>
</tr>
<tr>
<td>GATA4</td>
<td>++ +</td>
<td>++ +/-</td>
<td>++ +/-</td>
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</tr>
<tr>
<td>LHX9</td>
<td>++ +</td>
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</tr>
<tr>
<td>SOX9</td>
<td>- +</td>
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</tr>
</tbody>
</table>

**++** strong staining; + weak staining; – no staining; +/- variable positive staining

Abbreviations: CE, coelomic epithelium; SE, surface epithelium; med/IS, medullary/interstitial cells

*After E13.5, GATA4 is upregulated in Sertoli cells relative to interstitial cells in XY gonads and downregulated in all somatic lineages of the XX gonad.

Modified from (Barske et al., 2010).

1 (Armstrong et al., 1993); 2 (Pelletier et al., 1991); 3 (Albrecht et al., 2001); 4 (Tevosian et al., 2002); 5 (Jorgensen et al., 2005); 6 (Viger et al., 1998); 7 (Birk et al., 2000); 8 (Mazaud et al., 2002); 9 (Kobayashi et al., 2005)
complementary pattern of LHX9 and SOX9 indicates that as cells move into the medullary compartment (Yao et al., 2004) and begin to express SOX9, they may also be entering a more differentiated state.

Several RNA-level analyses have implied that SOX9 is expressed at medium-low levels in the bipotential turtle gonad and upregulated at the MPT after sex determination (Fig. 5A, (Spotila et al., 1998; Shoemaker et al., 2007; Shoemaker et al., 2007)). However, our immunocytochemical analysis revealed that the more dramatic and perhaps relevant event is the abrupt downregulation of the protein that occurs at the FPT at the end of the temperature-sensitive period (by stage 21; Fig. 3A). While a similar expression pattern was observed in the sea turtle Lepidochelys olivacea (Moreno-Mendoza et al., 1999; Moreno-Mendoza et al., 2001), this is not characteristic of all TSD species, as crocodilians, by contrast, only express SOX9 in the testis after sex determination (Western et al., 1999; Agrawal et al., 2009), similar to chicken (Oreal et al., 1998).

We provide evidence that adherens and tight junctions may act to maintain medullary cord structures during the bipotential period. At these early stages, β-catenin, E-cadherin, and ZO-1 were detected in medullary cord cells and the surface epithelium but not interstitial cells. At later stages, β-catenin was maintained in developing testis cords but absent from the medullary lacunae of differentiating ovaries. This pattern
suggests that adherens junctions disassemble as the medullary cords break down at the end of the TSP.

The emergence of the male-specific vasculature appears to be less critical for testis cord morphogenesis in the turtle as compared to the mouse. Not only are presumptive vessels evident at a relatively earlier stage in the XY mouse gonad, but chemical manipulations that inhibit vascular development also severely hinder cord formation and integrity (Combes et al., 2008; Cool et al., 2011). As the testis cords of the turtle gonad have already begun to expand and elaborate by stage 21, prior to the appearance of any endothelial-like cells within the gonad proper, we can conclude that at least the early events of testis cord morphogenesis in the turtle are not dependent on the existence of a vascular network within the gonad.

The precursors to adult granulosa cells, which are the supporting cells of the ovary, analogous to Sertoli cells, appear to only be generated in the differentiating ovary after the close of the TSP. These cells also arise from divisions in the coelomic epithelium (Pieau et al., 1999) but, unlike the medullary cord cells, remain in the cortex (Yao et al., 2004) and begin to form primitive follicle-like structures around meiotic oocytes by the hatching stage (Fig. 27; (Pieau et al., 1999)). Proliferation of these somatic cells and pre-meiotic oocytes accounts for the substantial increase in the size of the cortex in differentiating ovaries (Schmahl et al., 2003). We determined that increased somatic proliferation in the cortical domain was first apparent at stage 21, well after SOX9 had
been downregulated (~stage 19/20; Fig. 29A). In accord with this timeline, inhibition of proliferation did not interfere with SOX9 downregulation in cultured FPT gonads (Fig. 29B). These findings indicate that feminization of the medulla precedes and is independent of cortical expansion.

Presumptive turtle ovaries therefore produce Sertoli cell precursors during the TSP but then divert those cells to lacuna fate while generating a new set of granulosa cell precursors after sex is determined. To some extent, this pattern resembles the manner in which male and female supporting cells are specified in the mouse. Here, the bipotential supporting cell precursors give rise to all of the Sertoli cells in XY gonads, but only contribute to a limited subset of follicles in the ovary; most of the granulosa cells arise from the coelomic epithelium after sex has been determined, concomitant with follicle assembly (see section 3 and (Mork et al., 2011)). Furthermore, the ovarian lacuna cells, which derive from the ostensible Sertoli cell precursors, are steroidogenic, at least during embryonic stages (Fig. 31), and are likely involved in the maintenance of ovarian fate or the establishment of ovarian functionality at the hormonal level, similar to the purported role of the first wave of medullary follicles in the mouse (see section 3.4).

One of the more significant aspects of this work is that it provides a new context in which we can think about how temperature affects sex determination in this species. The lack of working markers to date has led the field to consider this phenomenon from the whole-gonad perspective rather than from the point of view of individual cell types; that
is, RNA level expression analyses and hormonal assays have only been done in whole
gonads, so the individual profiles of germ cells and each somatic cell type have not been
distinguished from each other. New markers and careful detailing of cellular events will
help to determine where the temperature-sensitive fate decision is actually made in this
species.
6. Mechanisms underlying estrogen’s feminizing effect on the turtle ovary

6.1 Summary

Despite the overwhelming evidence indicating that estrogen is critically required for female fate determination and ovary development in non-mammalian vertebrates, the downstream receptors and pathways through which estrogen exerts its effect on the gonad have not yet been elucidated. Estrogen’s impact on the reptilian gonad is thought to be local and not a consequence of distant effects on the brain (Pieau et al., 2004), though a previous study did not find the gonad to be a major site of estrogen uptake (Gahr et al., 1992). It has been difficult to separate the direct consequences of estrogen exposure from indirect effects related to female development in general. This section describes the results of a number of experiments that begin to address these issues. We investigated the distribution of aromatase protein expression, the involvement of canonical and non-canonical estrogen receptor activation, the effect of partial aromatase inhibition on ovary development, the impact of estrogen on several aspects of ovary differentiation, and a possible link between estrogen and Wnt signaling in the turtle ovary.

6.2 Introduction

Eutherian mammals have evolved a strictly genetic sex-determining mechanism compatible with placental development and exposure to maternal hormones (Wolf
1999). Through a poorly understood mechanism, eutherians evade the susceptibility to estrogen observed in most non-mammalian vertebrates and protect genetically male embryos from sex-reversing to phenotypic females. Egg-laying vertebrates are generally free from this constraint, and actually use estrogen as an endogenous feminizing signal (e.g., Pieau et al., 2004; Ramsey et al., 2008; Guiguen et al., 2009).

In many vertebrate species, with the notable exception of eutherian mammals, ectopic exposure to estrogen during the bipotential period of gonad development can override genetic or environmental signals and cause male-to-female sex reversal. The sensitivity of aquatic species to estrogen is evident in many populations exposed to high levels of environmental estrogens, where varying degrees of male-to-female sex reversal have been reported (reviewed in Matthiessen et al., 1998; Guillette et al., 2000; Edwards et al., 2006). As the steroidogenic enzyme aromatase, which converts testosterone to E2, is also known to be upregulated concurrently with the onset of ovarian differentiation (e.g., Desvages et al., 1992; Desvages et al., 1993; Chardard et al., 1995; Andrews et al., 1997; Smith et al., 1997; Ramsey et al., 2007; Jorgensen et al., 2008; Okada et al., 2009), it is likely that estrogen acts in the native pathway by which the bipotential gonad forms an ovary in these species (reviewed in Pieau et al., 2004). Indeed, regulation of aromatase expression has been proposed as the functional read-out of temperature in TSD species (Lance 2008).
Further evidence for a critical role of estrogen in female fate determination was provided by experiments involving pharmacological inhibition of estrogen synthesis. Treatment of fish, turtle (Crevis et al., 1994; Wibbels et al., 1994), lizard (Wibbels et al., 1994), newt (Chardard et al., 1999), frog (Olmstead et al., 2009), chicken (Elbrecht et al., 1992; Smith et al., 2003; Hudson et al., 2005) or alligator (Lance et al., 1992) embryos with aromatase inhibitors induced female-to-male sex reversal, with variable severity. Conversely, genetically female mice lacking the genes encoding both estrogen receptors (Esr1/Esr2) or aromatase (Cyp19a1) display normal early ovarian development with partial postnatal female-to-male sex reversal, indicating that estrogen signaling is not required for the initial commitment to ovarian development in the mouse gonad (Fisher et al., 1998; Couse et al., 1999). This may not be the case in all mammals, as genetically male marsupials can be sex-reversed by perinatal treatment with estradiol benzoate (Fadem et al., 1986; Coveney et al., 2001; Renfree et al., 2001), and a mutation affecting the transcription factor Foxl2 prevents the upregulation of aromatase in the developing goat ovary, resulting in a male phenotype (Pailhoux et al., 2005).

Experiments involving other steroid hormones or enzyme inhibitors further support a primary role for estrogen in determining female fate. Exposure to testosterone, for example, has no effect on FPT embryos but can induce female development in embryos incubating at the MPT or intermediate temperatures (Crevis et al., 1995). This effect is attributed to the aromatization of testosterone into E2. Administration of
androstenedione, a precursor to both testosterone and E2, produced a similar feminizing effect at the MPT as testosterone (Crews et al., 1995). All three forms of estrogen (17β-estradiol (E2), estrone (E1), and estriol (E3)) can induce female development at male temperatures, though with variable potency (Crews et al., 1995; Crews et al., 1996). The non-aromatizable form of testosterone, dihydrotestosterone (DHT), also has no effect on FPT embryos (Crews et al., 1989; Wibbels et al., 1992) but masculinizes embryos incubating at female-biased or intermediate temperatures (Wibbels et al., 1992). This masculinizing effect is specific to non-aromatizable androgens (Crews et al., 1996).

The sex-reversing effect of exogenous estrogen on non-mammalian vertebrates stems from a primary effect on the gonad rather than secondary effects on somatic organs. Embryos treated with E2 develop ovaries with a reduced medulla and a fully expanded cortex containing meiotic germ cells (e.g., Wibbels et al., 1993). Likewise, embryos exposed to aromatase inhibitors develop testes (e.g., Crews et al., 1994). Estrogen thus appears to affect both the cortex and medulla of the bipotential gonad and is probably involved in the endogenous pathway that synchronizes medullary reorganization and cortical expansion in the turtle ovary (Pieau et al., 2004).

Interestingly, when turtle embryos were exposed to lower doses of estrogen, nominally intersex hatchlings presenting ovotestes with testis cords and a fully expanded cortex were occasionally observed (reviewed in (Pieau et al., 1999)). Furthermore, when embryos of the European pond turtle, *Emys orbicularis*, were
incubated at temperatures midway between the MPT and FPT, hatchlings often presented ovotestes and showed intermediate levels of aromatase activity (Pieau et al., 1998). These findings suggest that low doses of estrogen are sufficient to stimulate cortical development, but female differentiation of the medulla requires a higher dose.

While the evidence supporting a critical role for estrogen in initiating ovary development is thus very strong, the downstream pathways through which it exerts its effects are unknown. The following section details our investigation of the site of estrogen production as well as several candidate mediators of estrogen signaling in the turtle ovary.

6.3 Results

6.3.1 Aromatase is expressed in the lacunae of differentiating turtle ovaries

The first step in untangling the estrogen signaling pathway in the turtle ovary is to identify the site of estrogen production. Aromatase expression is upregulated in FPT gonads at the end of the TSP (Ramsey et al., 2007), but it remains unclear where in the ovary it is expressed. We therefore immunostained stage 24 FPT gonads with an antibody that recognizes a conserved domain of the human aromatase protein. At this stage, aromatase expression was highly enriched in the lacuna cells (Fig. 31). This
Figure 31. Medullary aromatase expression in the differentiating turtle ovary.

Aromatase expression (green) was highly enriched in medullary lacunae of the differentiating ovary (stage 24). The dashed line indicates the border between the cortex and medulla. Nuclei were stained with syto13 (blue). The image was taken at 400×. Scale bars represent 20 μm.
Figure 31: Medullary aromatase expression in the differentiating turtle ovary
finding agrees with the aromatase expression patterns reported for fetal chickens (Hudson et al., 2005; Smith et al., 2008) and goats (Pannetier et al., 2006). It is still unclear, however, whether upstream members of the steroid biosynthesis pathway are also expressed in these cells, or even in the ovary at all.

6.3.2 Inhibition of the estrogen receptors delayed female differentiation

The gonadal cells that respond to estrogen signaling have not yet been identified. The two canonical estrogen receptors, ERα and ERβ, are known to be transcribed in the turtle gonad during the TSP and subsequent sexual differentiation (Bergeron et al., 1998; Ramsey et al., 2007), but attempts to determine their precise localization have not been definitive.

Nonetheless, activation of ERα and/or ERβ represents the most logical pathway through which estrogen could exert its effects on the gonad. To determine whether ERα or ERβ mediate the effect of estrogen on the gonad, we treated eggs incubating at the FPT with the pan-ER antagonist ICI182780 at three stages toward the end of the TSP (17, 19, and 20), and dissected at stages 21 and 26. Sex was evaluated by SOX9 expression and general gonad morphology. While all treated stage 26 hatchlings showed a normal ovarian phenotype, indicating that the drug had not induced sex reversal, four of the six embryos dissected at stage 21 showed residual SOX9 expression in clusters of medullary cells at the anterior and posterior ends of the gonad (Fig. 32C). As this pattern was never observed in control females at this stage (Fig. 32A, B), we conclude that the ICI182780
Figure 32. Inhibition of the estrogen receptors delayed female differentiation as judged by SOX9 expression. Eggs incubating at the FPT were treated with the pan-ER antagonist ICI182780 at three stages toward the end of the TSP (17, 19, and 20) and dissected at stage 21. SOX9 (yellow) was absent in control FPT gonads (A) as well as 2/6 gonads from ICI182780-treated embryos (B). However, 4/6 of the treated gonads showed delayed SOX9 downregulation at the anterior and posterior ends of the gonad (C). Nuclei were stained with syto13 (blue). Images of whole-mount immunostained gonads were taken at 400×. The scale bar represents 20 μm. Modified from (Barske et al., 2010).
Figure 32: Inhibition of the estrogen receptors delayed female differentiation as judged by SOX9 expression
treatment delayed the normal downregulation of SOX9 in these cells and their subsequent female differentiation. The subtle effect of this treatment might be attributed to dose or the molecular structure of the antagonist, which could have prevented it from efficiently penetrating the eggshell and embryo.

6.3.3 Evidence against a non-canonical estrogen pathway involving ERK signaling

The MAP kinase ERK1/2 becomes phosphorylated following the activation of a number of different receptors, though it is most frequently associated with receptor tyrosine kinase signaling. Relative to other stages and cell types, phosphorylated ERK (pERK) levels are consistently strongly elevated in the proliferating somatic cortical cells of the differentiating turtle ovary (Fig. 33A, B). Positive staining in other cell types was less consistent between samples. This proliferative event may be regulated by a canonical growth factor pathway such as the FGF pathway. However, estrogen signaling has also been linked to ERK phosphorylation via a non-canonical transmembrane estrogen receptor and PKC signaling [30-32]. Therefore, it is possible that the mechanism by which estrogen regulates cortical expansion involves direct ERK activation, which may preclude the requirement for a growth factor intermediary or ER-induced gene transcription. We therefore investigated whether exposure to E2 altered pERK levels in cultured turtle gonads. We hypothesized that pERK levels would increase rapidly in
Figure 33. Estrogen is unlikely to stimulate cortical activation through an ERK-mediated non-canonical pathway. pERK levels were evaluated in FPT (A) and MPT (B) gonads before and after sex determination. Erratic staining was observed at stages 15-19 in both FPT and MPT gonads. However, pERK staining was highly and consistently enriched in somatic cells in the expanding cortex in stage 21 and 26 ovaries. Strong staining was also observed in the tunica albuginea and scattered interstitial and Sertoli cells in the stage 21 and 26 testes. (C-F) Short-term exposure to E2 or E3 (estriol) did not appear to activate ERK signaling. MPT stage 18 gonads cultured for 1 h in control medium (C, E) or medium containing 500 ng/ml E2 (D) or E3 (F) showed no consistent changes in pERK levels or distribution. The dashed lines in the stage 21 and 26 panels of (A) represent the border between the cortex and medulla. The vertical grey line separates the bipotential stages from later sexual differentiation. Nuclei were stained with syto13 (blue). Images of whole-mount immunostained gonads were taken at 400×. The scale bar represents 20 μm.
Figure 33: Estrogen is unlikely to stimulate cortical activation through an ERK-mediated non-canonical pathway
response to E2 or E3 treatment if ERK was indeed being directly activated by a membrane-associated estrogen receptor.

MPT gonads (stage 17, 18, or 19) were cultured for 1-2.5 h in control medium or 1-500 ng/ml E2 (Fig. C, D) or estriol (E3; Fig. E, F) and immunostained for pERK. The staining intensity and number of pERK-positive cells were highly variable in both controls and treated samples, with no consistent changes observed in response to estrogen (Fig. 33C-F). Though the sensitivity of this assay was relatively low, these results indicate that estrogen probably does not directly stimulate the MAPK pathway in developing turtle gonads.

6.3.4 Partial aromatase inhibition caused embryos to develop ovotestes

As noted above, treatment of MPT turtle eggs with lower doses of E2 caused some embryos to develop with ovotestes. We aimed to perform the reverse experiment, i.e., to treat FPT eggs with lower or less potent doses of aromatase inhibitor that would lower but not eradicate the gonad’s supply of estrogen. This would allow us to confirm whether cortical expansion requires less estrogen than medullary cord reorganization and SOX9 downregulation. Hatchlings treated in this manner presented a full range of sexual phenotypes, including normal ovaries, ovotestes of variable proportions, and normal testes. The most extreme ovotestes (Fig. 34A) exhibited an expanded ovarian cortex containing primitive follicle structures and meiotic germ cells (SCP3-positive; Fig. 34B) as well as numerous morphologically normal testis cords in the medullary
Figure 34. Turtle ovotestes exhibit ovarian morphology and expression patterns in the cortex and testicular structures and expression patterns in the medulla. (A) Structure of turtle ovotestes. (B-E) Stage 26 ovotestes from FPT embryos treated with letrozole at stages 13, 15, 17 and 19. (B) Meiotic germ cells (SCP3-positive, magenta, asterisks; see inset for higher magnification) were only observed in the ovarian compartment; non-meiotic germ cells in testis cords are marked with arrowheads. (C) SOX9-positive Sertoli cells (yellow) were arranged into testis cords in the medulla. (D) WT1 (magenta) was expressed in the cortex as in a normal ovary and in the medulla as in a normal testis. (E) GATA4 (yellow) was strongly expressed in the ovarian compartment and enriched in Sertoli cells relative to the interstitial cells in the medulla, a pattern occasionally seen in control male gonads. Blue, nuclei stained with syto13. Images of whole-mount immunostained gonads were taken at 400×. AI, aromatase inhibitor (letrozole). Scale bars represent 50 μm. Modified from (Barske et al., 2010).
Figure 34: Turtle ovotestes exhibit ovarian morphology and expression patterns in the cortex and testicular structures and expression patterns in the medulla.
compartment. We examined the expression of WT1, GATA4, and SOX9 in the ovotestes and found that the patterns conformed to the ‘sex’ of each compartment (Fig. 34C-E). That is, each marker was distributed in the cortex as in normal ovaries (absent, in the case of SOX9), and in the medulla as in normal testes.

Importantly, the structure of these ovotestes indicates that expansion of the cortical region does not require a feminized medulla and that testis cord maintenance is not affected by the presence of an ovarian cortex.

### 6.3.5 Early exposure to estrogen induced premature downregulation of SOX9

Previous studies have shown that administration of a single dose of E2 at stage 17 is sufficient to induce male-to-female sex reversal (Crews et al., 1991), and medullary cord regression, a characteristic feature of ovarian differentiation, is apparent in these treated gonads by stage 20 (Wibbels et al., 1993), similar to the timing of this event in control FPT gonads. However, we hypothesized that by starting E2 treatments earlier than stage 17, we might prematurely induce estrogen-regulated features of ovarian differentiation at an earlier stage than controls. To test this idea, we applied E2 to turtle eggs incubating at the MPT early in the bipotential period (four doses given at stages 13, 15, 17 and 19) and analyzed changes in SOX9, WT1, GATA4, and LHX9 expression.

WT1, GATA4, and LHX9 expression were unaffected by early E2 treatment, echoing the patterns seen in control females (Fig. 35A-C). However, treatment with E2 induced
Figure 35. Changes in transcription factor expression patterns after estrogen treatment. Successive treatment of eggs with β-estradiol (E2) at the MPT (stages 13, 15, 17 and 19) did not affect WT1 (A, magenta), GATA4 (B, yellow), or LHX9 (C, magenta) expression, which followed the patterns seen in control female gonads. However, E2 induced precocious downregulation of SOX9 expression (D, yellow), such that no staining was observed in three out of four samples at stage 17 or in any stage 19 sample. Note that stage 15 samples received two doses of β-estradiol, stage 17 samples received three, and stage 19, 21, and 26 samples received four. The dashed lines demarcate the border between cortex and medulla. Blue, nuclei stained with syto13. Images of whole-mount immunostained gonads were taken at 400×. The vertical grey line separates the bipotentia stages from later sexual differentiation. The scale bar represents 20 μm. Modified from (Barske et al., 2010).
Figure 35: Changes in transcription factor expression patterns after estrogen treatment
premature repression of SOX9 expression in three of four stage 17 gonads and in all stage 19 samples examined (Fig. 35D). We measured SOX9 transcript levels by quantitative RT-PCR in E2-treated and control gonads and saw that SOX9 levels were consistently low in treated samples (Fig. 36A). The E2-treated gonads were quite small, as previously noted (Merchant-Larios et al., 1997), and had disorganized medullary cords throughout the bipotential period (Wibbels et al., 1993), but presented characteristic ovarian morphology by the hatching stage. The effect of estrogen on SOX9 protein expression was restricted to the bipotential period, as two doses of E2 applied to eggs incubating at the MPT around stage 23 (after sex determination) failed to elicit a similar response (Fig. 36B, C).

6.3.6 Evidence that Wnt signaling may be involved in establishing female fate in the turtle

Estrogen signaling might indirectly affect gonad development by modulating a downstream pathway or effector. One candidate mediator is the canonical Wnt signaling pathway. Several members of this pathway (e.g., Wnt4, Rspo1, β-catenin) have been shown to be critical for female development in mammals, with loss- or gain-of-function mutations resulting in a range of sex reversal phenotypes (Vainio et al., 1999; Parma et al., 2006; Chassot et al., 2008; Maatouk et al., 2008; Manuylov et al., 2008; Liu et al., 2009), including downregulation of SOX9 protein levels in XY gonads upon stabilization of β-catenin, the main downstream effector of this pathway (Maatouk et al., 2008). Estrogen
Figure 36. Estrogen signaling may regulate SOX9 downregulation in the differentiating ovary. (A) Quantitation of SOX9 mRNA levels in control FPT and MPT gonads suggests that SOX9 is sharply upregulated at the MPT after sex determination. We did not detect a significant, premature downregulation of SOX9 transcription in gonads successively treated with β-estradiol at the MPT (stages 13, 15, 17, and 19) relative to control FPT gonads (p > 0.05). Two to five pairs were analyzed per treatment/stage. Error bars represent SEM. MNE, mean normalized expression. (B) SOX9 expression (yellow) in stage 25 testes treated at stage 23 with ethanol (left panel) or β-estradiol (right panel). The repressive effect of estrogen on SOX9 protein expression appears to be restricted to the bipotential gonad, as later treatments had no detectable effect on SOX9 levels. Nuclei were stained with syto13 (blue). The scale bar represents 20 μm. Modified from (Barske et al., 2010).
Figure 36: Estrogen signaling may regulate SOX9 downregulation in the differentiating ovary
signaling also modulates the expression of Wnt ligands or β-catenin stability in the reproductive tract and neurons (e.g., Hou et al., 2004; Katayama et al., 2006; Varea et al., 2009), and β-catenin and SOX9 engage in a mutually antagonistic relationship in cultured chondrocytes (Akiyama et al., 2004), indicating that these pathways interact in multiple cellular contexts.

We first investigated whether the canonical Wnt pathway was involved in either the expansion of the cortex or the reorganization of the medulla in the developing turtle ovary. To ectopically activate Wnt signaling in MPT embryos, we cultured stage 17 and 19 gonads for six days with lithium chloride (LiCl), which stabilizes β-catenin by inhibiting GSK-3β (Klein et al., 1996). This treatment resulted in a nearly complete loss of SOX9 nuclear localization at both stages (Fig. 37). Because LiCl can also have off-target effects (Davies et al., 2000), we tested a second GSK inhibitor, GSK XV (Atilla-Gokcumen et al., 2006), and observed SOX9 downregulation as well as strong nuclear localization of β-catenin (data not shown). However, the drug induced considerable toxicity in the interstitium and germ cells, making the results difficult to interpret. No consistent ectopic cortical expansion was observed in any of these samples. These results indicate that stabilization of β-catenin is probably incompatible with nuclear SOX9 expression in turtle gonads.

Because both estrogen and ectopic Wnt activation had strong effects on the medullary domain, we hypothesized that Wnt might mediate some of estrogen’s effects
Figure 37. Stabilization of β-catenin induced SOX9 downregulation in MPT gonads.

*In vitro* treatment of MPT gonads (stage 17 or 19) with LiCl (25 mM) for six days induced downregulation of SOX9 (green) in medullary cord cells (highlighted with β-catenin staining, magenta) but no ectopic cortical expansion. The proportion of gonads with substantial nuclear SOX9 staining is noted in each panel. *In the five gonads deemed positive for SOX9 nuclear staining, very few positive cells were detected. Higher doses of LiCl induced complete SOX9 downregulation as well as considerable toxicity throughout the tissue (data not shown). Nuclei were stained with syto13 (blue). Images of cultured, immunostained gonads were taken at 200x. The scale bar represents 20 μm.
Figure 37: Stabilization of β-catenin induced SOX9 downregulation in MPT gonads
in the developing ovary. We first assessed whether manipulation of estrogen levels affected WNT4 expression. This gene presents similar expression levels in MPT and FPT gonads during the bipotential period, then becomes upregulated at the FPT by stage 21 (Shoemaker et al., 2007). A female-like pattern was observed in MPT samples exposed to estrogen in ovo, whereas FPT gonads treated with letrozole, an aromatase inhibitor (AI), presented a male-like pattern (Fig. 38A). This suggests that WNT4 transcription is activated downstream of estrogen signaling or repressed in its absence.

We next asked whether stabilization of β-catenin could rescue female development in FPT gonads exposed to an AI. We reasoned that if the β-catenin-induced antagonism of SOX9 was downstream or independent of estrogen, then co-treatment with LiCl and AI would result in SOX9 downregulation. If, however, stabilized β-catenin acted via estrogen to downregulate SOX9, we would observe maintenance of nuclear SOX9 expression when aromatase was inhibited. FPT gonads (stage 17) were cultured in control medium or medium containing AI, LiCl, or both AI and LiCl for six days (Fig. 38B). Some degree of nuclear SOX9 staining was seen in 15/24 gonads treated with AI alone, indicating that our in vitro sex reversal protocol was moderately effective. However, no nuclear SOX9 staining was observed in gonads cultured in control (n = 16), LiCl (n = 9) or AI + LiCl (n = 9) conditions, consistent with the experiments performed on MPT gonads. These findings collectively suggest that stabilization of β-catenin induces
Figure 38. The Wnt pathway may act downstream of estrogen during turtle ovarian development. (A) \textit{WNT4} expression is regulated by estrogen action. \textit{WNT4} expression was upregulated after stage 19 in gonads from control FPT embryos as well as MPT embryos treated with estrogen (E2). The relatively low levels of \textit{WNT4} in MPT + E2 samples might be associated with the growth defect observed in E2-treated gonads. In contrast, \textit{WNT4} levels declined after stage 19 in gonads from control MPT embryos and FPT embryos treated with an aromatase inhibitor (AI). MNE, mean normalized expression. Error bars indicate SEM. (B) Activation of Wnt signaling rescued some aspects of female development in the absence of estrogen. No SOX9 expression (green) was detected in cultured control or LiCl-treated FPT samples. Exposure to an AI induced female-to-male sex reversal and maintenance of SOX9 expression in 15/24 samples. Simultaneous inhibition of aromatase and activation of $\beta$-catenin with LiCl rescued SOX9 downregulation, suggesting that $\beta$-catenin’s effect on SOX9 is downstream of the feminizing effect of estrogen. The proportion of gonads with substantial nuclear SOX9 staining is noted in each panel. Nuclei were stained with syto13 (blue). Images of cultured, immunostained gonads were taken at 400$\times$. The scale bar represents 20 $\mu$m.
Figure 38: The Wnt pathway may act downstream of estrogen during turtle ovarian development
SOX9 downregulation in the medullary domain downstream or independently of estrogen.

In a final complementary experiment, we treated FPT gonads with one of two Wnt inhibitors, XAV939 or IWR-1, which both inhibit tankyrase, indirectly stabilizing Axin and promoting β-catenin destruction (Chen et al., 2009; Huang et al., 2009; Karner et al., 2010). Because ectopic activation of Wnt signaling suppressed SOX9 expression in MPT gonads, we predicted that inhibiting the pathway in FPT gonads might cause SOX9 to be maintained. However, neither FPT stage 18 gonads exposed to XAV939 for ten days nor FPT stage 17 gonads treated with IWR-1 for ten days retained SOX9 expression or presented reduced cortical expansion or β-catenin staining (Fig. 39). Though these results argue against a role for Wnt signaling in these processes, they could also be attributed to an insufficient dose or a failure of the drugs to recognize the turtle homolog of their target protein, and we therefore cannot rule out the possible involvement of the Wnt pathway in these or other aspects of ovarian differentiation.
Figure 39. Inhibition of Wnt signaling in cultured FPT gonads had no impact on SOX9 expression or cortical expansion. The Wnt inhibitors XAV939 and IWR-1 were applied at 2 μM and 10 μM, respectively. The proportion of gonads with substantial nuclear SOX9 staining (green) is noted in each panel. β-catenin immunostaining (magenta) was used to highlight the cortex of differentiating ovaries. Dashed lines indicate the border between the cortex and medulla. Nuclei were stained with syto13 (blue). Images of cultured, immunostained gonads were taken at 400×. The scale bar represents 20 μm.
Figure 39: Inhibition of Wnt signaling in cultured FPT gonads had no impact on SOX9 expression or cortical expansion.
6.4 Discussion

Although the feminizing influence of estrogen on the gonads of non-mammalian vertebrate embryos was first demonstrated many decades ago (Dantchakoff 1937), the downstream receptors and pathways that mediate this effect have not yet been determined. In the experiments described in this section, we attempted to identify and/or rule out candidate mediators of estrogen signaling in developing turtle gonads. These studies should be considered preliminary because, in many cases, the tools used to perturb and evaluate the system were not optimal. Nonetheless, our findings lend some insight into the means by which estrogen promotes ovarian development.

We provide evidence that estrogen acts to suppress testis differentiation at the FPT by repressing SOX9 expression in the medulla. E2 treatment caused premature suppression of SOX9 (Fig. 35D) and dissolution of cord structures in the medulla (Wibbels et al., 1993), while mild aromatase inhibition maintained SOX9 and testis cords and resulted in ovotestis development (Fig. 34). It is possible that the estrogen receptors directly repress transcription of SOX9 in this system, as inhibition of estrogen receptor signaling at the FPT using the ER antagonist ICI182780 delayed SOX9 downregulation in medullary cells at the anterior and posterior ends of the gonad (Fig. 32). This treatment did not induce full female-to-male sex reversal in hatchlings, but more effective antagonists may elicit a more comprehensive effect.
SOX9 is necessary and sufficient for commitment to the testis fate in mice (Qin et al., 2004; Kobayashi et al., 2005; Qin et al., 2005; Barrionuevo et al., 2006), and its association with the male pathway is extremely well conserved across vertebrates (Morrish et al., 2002). Our result aligns with studies in the mouse that detected a similar antagonistic relationship between estrogen signaling and SOX9, in which mutations in aromatase or both ERs induced upregulation of Sox9 and transdifferentiation of adult granulosa cells into Sertoli-like cells (Couse et al., 1999; Dupont et al., 2003; Britt et al., 2004). ER binding sites have been detected in the human SOX9 promoter (Jin et al., 2005; Ramsey et al., 2008), and ERα binds to and suppresses the murine Sox9 promoter in conjunction with FOXL2 in adult granulosa cells (Uhlenhaut et al., 2009). While further experiments are needed to demonstrate a direct inhibitory relationship in the turtle, these results collectively point toward a conserved role for estrogen signaling in the repression of SOX9 expression in the ovary. Given the well-characterized functional importance of SOX9 in human and mouse testis differentiation, it seems likely that its expression is incompatible with normal ovarian development.

Estrogen is clearly necessary and sufficient for cortical expansion in the turtle ovary. Because the proliferating somatic cells in the cortex exhibit high levels of pERK, we hypothesized that estrogen may act through a non-canonical pathway and stimulate proliferation via the ERK pathway. Our results, however, do not support this hypothesis and instead indicate that a different mitogen likely regulates pERK levels. Future
experiments should investigate the effects of ERK inhibitors to better elucidate the role of this pathway in turtle ovarian development.

Estrogen’s effect on cortical cells may be stage-dependent, as exposure to estrogen early in the bipotential period did not induce premature cortical expansion (Fig. 35). This conclusion is in line with the pattern of aromatase expression in the ovary. Aromatase is not upregulated in the ovary until the end of the TSP (Ramsey et al., 2007) and then is restricted to the medulla (Fig. 31), suggesting that cortical cells may not be competent to respond to estrogen signaling during early stages of gonad development. This distribution of aromatase also supports the idea that an estrogen gradient exists within the ovary, with higher concentrations in the medulla relative to the cortex, which in turn could explain why lower levels of estrogen appear to be needed to activate cortical expansion than to direct the reorganization of the medullary domain (Pieau et al., 1998).

We also investigated whether the canonical Wnt pathway played a conserved role in turtle ovary development, independent of or related to estrogen. We show that SOX9 expression in the turtle gonad responds to ectopic activation or inhibition of the canonical Wnt pathway in the same way as the mouse. We observed extremely robust SOX9 downregulation in response to β-catenin stabilization at the MPT (Fig. 37), suggesting that activation of the canonical Wnt signaling pathway antagonizes SOX9 expression or protein stability. This result is in accordance with previous work in the
mouse, where both treatment of XY gonads with LiCl and Cre-induced expression of a stabilized form of β-catenin resulted in SOX9 downregulation (Maatouk et al., 2008). Because *in vitro* stabilization of β-catenin had a similar repressive effect on SOX9 expression as early exposure to β-estradiol, we reasoned that Wnt signaling might act downstream of estrogen to promote SOX9 downregulation and female development. One way to test this idea would be to culture MPT gonads with estrogen plus a Wnt inhibitor, predicting that SOX9 expression would remain high in the absence of Wnt signaling, despite the presence of estrogen. However, given our uncertainty about the efficacy of the available Wnt inhibitors in turtle tissues, we chose to perform the opposite experiment, in which FPT gonads were cultured with LiCl and an aromatase inhibitor. This treatment resulted in complete SOX9 downregulation (Fig. 38), consistent with the idea that β-catenin acts downstream of estrogen to repress SOX9 expression.

We were unable to demonstrate an unequivocal endogenous role for Wnt signaling in ovarian development, as FPT gonads exposed to two different Wnt inhibitors were indistinguishable from controls (Fig. 39). We conclude that this pathway is either not required for early ovarian development in the turtle or was insufficiently repressed by the inhibitors due to a deficiency in the dose or target recognition. The latter possibility is somewhat unlikely, at least for IWR-1, because this compound has been shown to exert potent effects in zebrafish as well as mammals.
However, Wnt signaling may still be involved in the repression of other aspects of testis differentiation in turtles. In the mouse, loss of β-catenin, Wnt4, or Rspo1 results in a partial female-to-male sex reversal phenotype that involves the production of testis-specific vasculature and ectopic steroidogenesis but not robust SOX9 expression (Vainio et al., 1999; Jeays-Ward et al., 2003; Kim et al., 2006; Parma et al., 2006; Chassot et al., 2008; Liu et al., 2009). The mechanism underlying the discrepant effects of β-catenin loss- and gain-of-function mutations on SOX9 levels has not yet been explained. However, even if inhibition of Wnt signaling induced a phenotype in turtle gonads similar to that observed in mice, it would be difficult to detect this using either of the reported culture techniques, as the mouse phenotype involves ectopic cell migration from neighboring tissues, and it is extremely difficult to culture intact turtle gonad-mesonephros complexes.

Though an endogenous role for estrogen in turtle ovary development appears to be almost incontrovertible, there has long been one weakness in the model, namely, that estrogen levels in the gonad are nearly undetectable. While aromatase mRNA (Ramsey et al., 2007), protein (Fig. 31), and catalytic activity (Desvages et al., 1992) are all demonstrably present in the developing turtle ovary, many assays of other steroid hormones and steroidogenic enzymes have indicated that the gonads are not steroidogenically active tissues, especially in comparison to the neighboring adrenal gland and mesonephros. Most of the sex- or temperature-specific differences observed
during the TSP were also small or inconsistent. For example, 17β-HSD, 3α-HSD, and 3β-HSD activities were detected in the adrenals and mesonephroi but not the gonads of stage 17 and 26 *T. scripta* embryos (Thomas et al., 1992). In another study, secretion of progesterone and corticosterone from adrenal-kidney-gonad complexes (AKGs) was robust during and after the TSP, whereas secretion of androstenedione, dehydroepiandrosterone, and testosterone was only observed after the TSP, with the former two slightly higher in FPT gonads and the latter at the limit of detection in both sexes (White et al., 1992). Although very low levels of E2 were detected in secretions from FPT stage 21 AKGs, neither progesterone, corticosterone, testosterone, nor E2 were secreted from isolated gonads, indicating that the ‘AK’ component of the complex performed most of the steroid biosynthesis (White et al., 1992). Another study determined that the major sites of estrogen uptake in *T. scripta* embryos were the mesonephros and oviduct, not the gonad (Gahr et al., 1992), though later studies showed that both canonical estrogen receptors are expressed in both MPT and FPT gonads (Ramsey et al., 2007).

Studies of the European pond turtle *Emys orbicularis*, another TSD species, have yielded more interpretable and consistent results. One study detected male-enriched 3β-HSD activity in the medullary cord cells of undifferentiated and differentiated gonads (Pieau 1974), while a follow-up study showed that both FPT and MPT gonads could actively metabolize steroid precursors at multiple stages of development, with sex-
specific differences in conversion rates and dominant metabolites (Desvages et al., 1991). One final study found slightly elevated levels of E2 in developing ovaries (Dorizzi et al., 1991). As no direct species comparisons have been performed in a single study, it is unclear whether these species differences are reflective of true functional differences or technical inconsistencies. Collectively, these reports of steroidogenesis in turtle gonads are inconclusive at best, and it remains to be determined whether low levels of steroidogenesis in the gonad produce sufficient quantities of steroid precursors to generate the estrogen needed for female development or whether said precursors derive from other tissues.

Two lines of evidence, however, have refuted the hypothesis that signals arising from non-gonadal tissues regulate the temperature-sensitive decision. First, isolated gonads sex-reverse in response to shifts in incubation temperature, similar to embryos in ovo, albeit with a slight delay (Moreno-Mendoza et al., 2001; Shoemaker-Daly et al., 2010). This indicates that gonads can interpret changes in temperature without input from other tissues. With respect to hormones, treatment of cultured FPT gonads with an aromatase inhibitor induced male development in a subset of samples (Fig. 38B), indicating that gonadal, not brain, aromatase protein is the functional target of the inhibitor.

Though the results from in ovo experiments involving aromatase inhibitors, testosterone, and DHT support a primary role of E2 in female fate determination and a
potential role for non-aromatizable androgens in testis development, the extremely low levels of these hormones in the *T. scripta* gonad still present an obstacle for the success of this model. It is possible that other steroids present at relatively higher levels in the gonad may also influence sex determination. A derivative of dihydrotestosterone (DHT), 5α-androstane-3β, 17β-diol (3β-adiol), was recently been shown to be a potent ligand for ERβ (Guerini et al., 2005; Oliveira et al., 2007). This steroid, though derived from androgens, does not bind to the androgen receptor or have androgenic activity (Guerini et al., 2005). 3β-adiol can be produced from DHT in a reaction catalyzed by 3β-HSD or from androstenedione in a three-step reaction. 3β-adiol is subsequently converted to a water-soluble form by CYP7B1, which abolishes its estrogenic signaling activity (Pettersson et al., 2010). Interestingly, one of the sex-specific differences in *E. orbicularis* steroid metabolism involves this hormone. In differentiating ovaries, androstenedione was predominantly metabolized to 3β-adiol, whereas in testes, it was converted to 5α-androstane-3α,17β-diol (3α-adiol) (Desvages et al., 1991). This result is consistent with a role for 3β-adiol in ovarian estrogen signaling.

It is unclear whether ERα or ERβ mediates the effects of estrogen on the reptilian gonad. These receptors present many non-overlapping functions in mammalian systems. Though both genes are expressed in the turtle gonad, they may show different cell type-specific patterns and thus be poised to mediate different activities. It would be very interesting if, for example, the disparate effects of estrogen on cortical and
medullary development were mediated by different receptors. Future studies should take advantage of the many isoform-specific agonists and antagonists developed in recent years to explore this question.
7. Conclusions and future directions

The work presented in this dissertation has clarified the origins of granulosa cells in the mouse ovary and revealed that the bipotential supporting cell precursors are mitotically arrested during their initial specification. Our work adds complexity to the simple bifurcation model, which postulates that supporting cell precursors in the murine bipotential gonad give rise either to adult Sertoli cells or adult granulosa cells. However, our results also reveal several unexpected similarities between the male and female supporting cell lineages. First, precursors of the adult supporting cell lineages are generated from the surface epithelium up until the point at which the supporting cells and germ cells organize into their functional units – the testis cords in the male and the follicles in the female – and become segregated from the rest of the cells in the organ by a layer of basement membrane. Second, these precursors are mitotically arrested from their earliest specification until approximately the time at which their resident follicle or testis cord begins to expand. Viewed from this perspective, Sertoli and granulosa cell precursors actually arise in a similar manner, with the ostensible differences mostly attributable to the delay in follicle assembly relative to testis cord formation.

In the mouse, follicle formation is thought to be suppressed by high levels of maternal estrogen (Jefferson et al., 2006; Chen et al., 2007). Follicles begin to form immediately after birth, when this inhibitory signal is removed, and can be induced to assemble earlier when ovaries are removed from late-stage embryos and cultured in
vitro (Chen et al., 2007). Culturing perinatal ovaries with estrogen disrupts follicle formation and results in the formation of multiocyte follicles (Chen et al., 2007; Chen et al., 2009). It is possible that the inhibitory signal that delays follicle formation until birth evolved to prevent follicles from inappropriately responding to high levels of circulating maternal estrogen in a manner akin to follicles present in the ovary of a physiologically mature, postpubertal mouse.

In a complementary project on the red-eared slider turtle, we revealed evolutionary conservation in the molecular identity and relative positions of cell types present in developing mouse and turtle gonads. We also evaluated the potential involvement of a number of candidate pathways that might mediate the effect of estrogen on the turtle gonad. We speculate that estrogen production by the lacuna cells in the medulla of the differentiating ovary directs the downregulation of SOX9 in these same cells upon commitment to the female fate.

We identified an instance of evolutionary conservation between mice and turtles in that both species specify the Sertoli cell population during the bipotential period but generate granulosa cells after sex determination. In turtles, the whole population of granulosa cells appears to be generated in the coelomic domain after sex determination, whereas in mice, the supporting cell precursors, specified during the bipotential period, give rise to granulosa cells in medullary follicles, while cortical granulosa cells arise from coelomic domain after sex determination. We also propose that the lacuna cells in
the medulla of the turtle ovary and the medullary follicles of the early postnatal mouse ovary may have similar functions – both are steroidogenic and may be involved in establishing ovarian hormonal functionality.

These studies collectively indicate that many of the seemingly vast differences between species and/or sexes in how the gonads form or how sex is determined may in reality be illusory or artifacts of flawed paradigms.

7.1 Establishment of the supporting cell lineage

The problem of how neighboring cells adopt different fates applies to nearly every organ in a developing embryo and remains an open question with respect to the bipotential gonad. Our experiments to date have not definitively identified the factors that distinguish the bipotential supporting cell lineage from the rest of the somatic cells in the gonad. These cells, found throughout the early gonad underneath the coelomic epithelium, are mitotically arrested and competent to express Sry, but it is unclear whether these properties are relevant to the establishment of the lineage. Microarray analyses have identified a number of other genes specifically expressed by these cells during the bipotential period, any one of which may turn out to be the elusive lineage-restricting factor. However, no studies have succeeded in comparing the transcriptomes of the supporting cell lineage and the coelomic epithelium. This epithelium appears to be the major source of the supporting cell precursors (Fig. 8; (Karl et al., 1998)), but it differs from its descendants in gene expression patterns (e.g., Bmp2 (Yao et al., 2004) and
Developing a marker for the coelomic epithelium that is amenable to fluorescence-activated cell sorting for microarray analysis is an important goal for future work on this problem, as it would facilitate in-depth comparisons of the two populations and the identification of significant differences between them.

Our preliminary analysis of the Numb/Numbl mutant gonads suggests that the Notch pathway, or antagonism thereof, may act to restrict the lineage of supporting cell precursors as they exit the coelomic epithelium (Fig. 18). Confirmation of these results would extend our previous finding that antagonism of Notch signaling is required for the differentiation of Leydig cells (Tang et al., 2008) to gonadal cells in general. Ongoing experiments to address these issues are detailed in section 4.3.2.1.

We also plan to investigate the effects of expanding or reducing the number of arrested cells in the bipotential gonad (see section 4.4 for details). These experiments will more definitively determine whether cell cycle arrest is a prerequisite for adopting supporting cell fate. If so, the bipotential gonad could serve as an interesting model in which to study the intersection between cell fate decisions and the cell cycle. We hypothesize that arresting the cell cycle permits epigenetic changes required to adopt supporting cell fate. One line of work will address the possibility that cell cycle arrest is integrated with genome reprogramming in supporting cell precursors, perhaps through direct relationships between proteins in the polycomb complex and negative regulators.
of the cell cycle. For example, pRb can associate with multiple transcription complexes known to activate or repress target genes (Korenjak et al., 2005) as well as with chromatin modifying enzymes (Jullien et al., 2008). Most interestingly, pRb has been shown to colocalize with polycomb complexes associated with nuclear targets (Dahiya et al., 2001), providing a strong link between cell cycle arrest in G0/G1 and polycomb-mediated control of cellular differentiation.

7.2 Transcriptome analysis of developing turtle gonads

Studies of sex determination and gonad development in TSD species have been limited to date by the difficulties associated with performing genetic or in vitro experiments in these organisms as well as the paucity of available sequence information. While several features specific to these model systems, e.g., the extent of the thermosensitive window and hormonal sensitivity, have been well characterized, many others remain unexplained, including the identity of the temperature-sensitive trigger(s). These shortcomings likely stem from our reliance on extrapolating from the mammalian system. While this approach has revealed a high degree of evolutionary conservation in the development of the vertebrate gonad (see section 5), it is not suitable for identifying novel factors specific to TSD.

We decided to address this problem with an unbiased approach designed to capture temperature-dependent differences in gene expression before and after sex determination. We pooled gonads from ~20 MPT and FPT embryos at stages 17 and 21
and subjected them to RNA sequencing on an Illumina HiSeq 2000 system. This direct sequencing method circumvents the need for an assembled *T. scripta* genome, as the transcriptomes can be assembled *de novo* or using the *Chrysemys picta* or *Gallus gallus* genome as a reference. With these data in hand, we will determine differences in transcript abundance at the two temperatures and two stages, and hopefully identify new candidate regulators of sex determination and ovary/testis development in this TSD system.
Appendices

In this section, I present the findings of two additional projects, neither of which is closely related to the main thesis. The first addresses the question of whether the BMP signaling pathway plays a significant role in the development of the mouse ovary, while the second tackles the long-standing question of how germ cell clusters arise in the fetal gonad.
Appendix A: BMP signaling in the fetal ovary

8.1 Summary

Although a number of BMP ligands are enriched in the mouse ovary at fetal stages, a role for BMP signaling in the development of this organ has not yet been definitively demonstrated. We took advantage of a BMP reporter line in order to gain insight into the cell-type specific patterns of BMP signaling during fetal gonad development. Reporter expression was specifically enriched in the ovary relative to the testis after sex determination and became specific to granulosa cells after birth. No reporter activity was observed in germ cells at any stage of development. BMP signaling in the fetal ovary is regulated by Wnt4 and Foxl2. In a preliminary analysis of mice in which BMP signaling was constitutively activated in gonadal somatic cells, no apparent defects in testis or ovary differentiation were observed. Future studies will drive constitutive BMP signaling in germ cells and exploit a loss-of-function model to further define the role of this pathway in ovary development.

8.2 Introduction

BMP signaling regulates a wide variety of developmental events, from bone formation and neuronal differentiation to primordial germ cell specification and follicle development in the adult ovary. BMP ligands bind to serine-threonine kinase type II BMP receptors (e.g., BMPR2), which then bind and phosphorylate type I receptors
(BMPR1A and BMPR1B). The activated receptors subsequently phosphorylate the receptor-associated SMAD1/5/8 proteins, allowing them to associate with SMAD4 and manipulate gene expression in the nucleus.

In the adult mouse ovary, BMP ligands and receptors are expressed in a complex pattern that reflects their important role in communicating information between oocytes, granulosa cells, and theca cells. For example, oocyte-derived GDF9 (a related TGFβ ligand) and BMP15 both act to inhibit premature luteinization of granulosa cells, but Gdf9 is also specifically required for follicle growth past the primary stage, and Bmp15 is involved in ovulation (Otsuka et al., 2011; Paulini et al., 2011). Two other ligands, Bmp4 and Bmp7, have been proposed to regulate the transition of a primordial follicle to the primary stage (Lee et al., 2001; Tanwar et al., 2008), and Bmp5, expressed by granulosa cells, regulates proliferation and inhibits luteinization in an autocrine manner (Pierre et al., 2005). The expression patterns and effects of these ligands on ovary development and function vary between mammalian models (Juengel et al., 2006; Sun et al., 2010; Tanwar et al., 2011), but the role of BMP signaling in the fetal gonad is even less clearly defined and more controversial.

### 8.2.1 Germ cells are putative targets of BMP signaling in the gonad

BMPs (2, 4, 8b) induce the formation of primordial germ cells (PGCs) within the proximal epiblast at E7.5 in the mouse (Saitou 2009). The PGCs then migrate via the gut to arrive at the developing gonads by E10.5 (Lawson et al., 1994; Molyneaux et al.,
Several studies have shown that BMPs may help to guide PGC migration (Dudley et al., 2007) and regulate their proliferation and/or survival once they arrive in the gonad, though the results have been somewhat contradictory. For example, BMP4 promoted the proliferation of isolated E11.5 PGCs cultured on feeder layers (Pesce et al., 2002), but female E11.5 gonads cultured adjacent to beads coated with BMP2 or BMP4 presented reduced numbers of meiotic germ cells (Ross et al., 2003). Another group argued that germ cells are the sole target of BMP signaling in the human fetal ovary based on their observation that BMP type I receptors and pSmad1/5/8 were specifically expressed in germ cells (Childs et al., ). They also observed that ovaries cultured with BMP4 displayed an increase in apoptosis (Childs et al., ). Finally, a recent study of mice mutant for Msx1 and/or Msx2, known targets of both the BMP and Wnt pathways (e.g., Hussein et al., 2003; Brugger et al., 2004), revealed that these genes are enriched in PGCs and appear to promote PGC entry into meiosis (Le Bouffant et al., 2011).

These studies collectively argue that germ cells are likely a target, if not the only target, of BMP signaling during ovary development. However, in the mouse, BMP receptors are expressed in multiple cell types between E11.5 and E13.5, as described in more detail below (Fig. 40), and contributions of somatic cell types to the aforementioned phenotypes cannot easily be ruled out.
8.2.2 Evidence supporting a role for BMP signaling in ovary development

The effects of BMP signaling on germ cell specification and migration occur independently of sex. However, several studies have suggested that the BMP pathway may also play a sex-specific role in the development of the ovary. Bmp2 was one of the first genes shown to be expressed in an ovary-specific manner during the early stages of gonad development (Yao et al., 2004). Immunostaining (Dudley et al., 2007), in situ (Yao et al., 2004), qRT-PCR (Kashimada et al., 2011), and microarray (Jameson et al., unpublished; Fig. 40A) analyses collectively determined that Bmp2 was expressed in both sexes at E10.5, became specifically enriched in ovarian somatic cells (pregranulosa and stromal) by E11.5, and then declined after E13.5. Another BMP ligand, Bmp5, is also specifically enriched in female pregranulosa cells between E11.5 and E13.5 (Fig. 40A). Bmp4, Bmp6, and Bmp7, however, are expressed at similarly low levels in both sexes within this time window (Fig. 40A and data not shown). BMP receptors are also expressed in the gonad, though not in sexually dimorphic patterns. Bmpr1a is highly expressed in all cell types except endothelial cells, whereas Bmpr2 is strongly expressed everywhere except germ cells (Fig. 40B). These patterns of receptor-ligand expression and sexual dimorphism differ between model species (Quinn et al., 2004; Abir et al., 2008; Wang et al., 2009; Carre et al., 2011) and likely change throughout the course of ovary development.
A limited amount of data is available on the expression of BMP antagonists in the gonad. One putative agonist, *Crim1*, which is a transmembrane protein with chordin-like repeats in its extracellular domain, is specifically upregulated in Sertoli cells after E13.5 but is undetectable in the ovary throughout fetal development (Fig. 40C; (Georgas et al., 2000)). Two other antagonists, *Grem2/Prdc* and *Sostdc*, are upregulated in Sertoli and male interstitial cells starting at E11.5 and E12.5, respectively (Fig. 40C; (Nef et al., 2005)). *Twsg1* is expressed at very low levels in all cell types between E11.5 and E13.5, and no data is presently available for *Chrd*, *Nog*, *Grem1*, *Dand5*, *Cer1*, *Dan*, or *Sost*. The male bias in expression of three out of the four BMP antagonists for which data are presently available is striking and suggests that the testis may actively suppress BMP signaling during fetal development.
Figure 40. Expression of BMP ligands, receptors, and antagonists in early XX and XY gonads. Microarray data from four sorted cell populations (supporting cells (Sertoli and pregranulosa cells), interstitial/stromal cells, endothelial cells, and germ cells) collected from XX and XY gonads at E11.5-13.5 (Jameson et al., unpublished data). Dotted lines represent cell populations from XX samples, and unbroken lines represent cell populations from XY samples. Bmp2 and Bmp5 are highly enriched in female pregranulosa cells (blue dotted lines), whereas Bmp4 expression is low in all cell types. The type I receptor Bmpr1a is highly expressed in all cell types except endothelial cells (red lines), whereas the type II receptor Bmpr2 is strongly expressed everywhere except germ cells (green lines). Three BMP antagonists, Crim1, Grem2, and Sostdc1 are upregulated in XY Sertoli or interstitial cells relative to all female cell types after E11.5 (Grem2 and Sostdc1) or E12.5 (Crim1).
Figure 40: Expression of BMP ligands, receptors, and antagonists in early XX and XY gonads
8.2.3 *Bmp2* is downstream of *Wnt4* and *Foxl2* in the fetal ovary

The canonical Wnt signaling pathway is critical for the determination of female fate at the end of the bipotential window of gonad development. Wnt signaling inhibits the migration of the mesonephric endothelial cells that form the coelomic vessel in the testis, prevents the expression of steroidogenic enzymes, and promotes the survival of germ cells after E15.5 (Vainio et al., 1999; Jeays-Ward et al., 2003; Yao et al., 2004). Female gonads with mutations in *Wnt4*, *Rspo1*, or *Ctnnb1* (β-catenin) are partially masculinized but do not form testis cords, express substantial levels of SOX9, or undergo male-specific proliferation (Vainio et al., 1999; Jeays-Ward et al., 2003; Kim et al., 2006; Parma et al., 2006; Chassot et al., 2008; Manuylov et al., 2008; Liu et al., 2009). *Foxl2* expression is also maintained (Maatouk et al., unpublished data). Germ cell death in these mutants appears to occur in a wave beginning at the anterior pole of the ovary (Maatouk et al., unpublished data). Near birth, small cord-like structures expressing *Foxl2* and *Amh* form in the germ-cell depleted region ((Vainio et al., 1999); Maatouk et al., unpublished data).

Though the phenotype of the *Wnt4*, *Rspo1*, and *Ctnnb1* mutants suggests that the pathway plays a more active role in repressing aspects of testis differentiation than promoting ovarian development, several ovary-enriched genes, including *Bmp2* and *Fst*, are significantly downregulated in the absence of *Wnt4* (Yao et al., 2004). *Fst* mediates *Wnt4*’s inhibitory effects on the vasculature and germ cell survival by repressing activin-βb activity (Yao et al., 2004; Liu et al., 2010). Regulation of *Bmp2* by Wnt signaling,
however, is independent of Fst, as Bmp2 expression is maintained in the Fst mutant (Yao et al., 2004).

Bmp2 also appears to be regulated by FOXL2. In Foxl2−/− ovaries, Bmp2 expression is fairly normal at E13.5 but declines to about 60% of wild-type levels at birth (Kashimada et al., 2011). Loss of both Wnt4 and Foxl2 resulted in a synergistic decline in Bmp2 levels relative to the single mutants at both E13.5 and P0 (Kashimada et al., 2011). Exogenous BMP2 also promotes Fst expression in cultured granulosa cells and explanted gonads (Kashimada et al., 2011), though the in vivo relevance of this relationship remains unclear.

**8.2.4 Development of a BMP reporter**

Phosphorylated-SMAD1/5/8 levels have frequently been used as a reporter of BMP signaling activity. To date, pSMAD1/5/8 levels have been evaluated postnatally and during the earliest stages of gonad development (E9.5-11.5) but not during later fetal stages. In the latter, pSMAD1/5/8 was detected in the epithelium overlying the urogenital ridge and the PGCs that had entered the gonad as well as other somatic cells (Dudley et al., 2007).

Several years ago, Blank et al. (2008) developed a transgenic mouse line that reports BMP signaling (BRE-lacZ) (Blank et al., 2008). The reporter construct was made using BMP response elements (BREs) taken from the promoter of the Id1 gene, which is a well-known early target of BMP signaling. This particular series of response elements is
distinct from most BREs in that it can be activated by SMAD binding alone, in the absence of the cofactors that are usually required for SMAD DNA binding specificity. Therefore, expression of the lacZ reporter in a given tissue does not necessarily indicate that other BMP target genes would also be expressed, as these might require cofactors that are not expressed in the tissue. However, the authors showed that the β-galactosidase expression pattern correlated well with that of pSMAD1/5/8, indicating that the reporter accurately captures activation of the pathway (Blank et al., 2008).

8.3 Results

8.3.1 BRE reporter activity is enriched in fetal and postnatal ovaries

We evaluated the extent of BMP signaling in fetal gonads using the BRE-lacZ reporter. At E11.5-11.75, X-gal activity was detected in the gonads of both sexes (Fig. 41A, B). Immunostaining for β-galactosidase revealed specific staining in endothelial cells and scattered GATA4-positive somatic cells located near the top and middle of the gonad. However, no reporter activity was observed in the outermost coelomic domain or in the germ cells (Fig. 42A). This bias away from the most basal layers of the ovary and the lack of activity in the coelomic epithelium are reminiscent of the Bmp2 expression pattern determined by in situ hybridization (Yao et al., 2004). At E12.5, X-gal staining was stronger in the ovary than the testis (Fig. 41C, D). Endothelial cells were β-galactosidase-positive in both sexes. In the ovary, strong reporter activity was detected in many GATA4-positive cells, partially overlapping with the FOXL2-positive
population. Fewer β-galactosidase-positive cells were observed at the base of the ovary, and the coelomic epithelium remained negative (Fig. 42B). In the testis, weak reporter activity was observed in a subset of Sertoli cells as well as the aforementioned endothelial cells (Fig. 42F).

In E13.5-17.5 ovaries, strong activity was observed in FOXL2-positive cells (Fig. 42C). Reporter activity in endothelial cells declined throughout this period, and no staining was observed in the stromal compartment or the coelomic epithelium (Fig. 42C′, arrowhead). In the testis, strong activity persisted in endothelial cells throughout fetal stages, and a number of β-galactosidase-positive Leydig cells were observed at E15.5 (Fig. 42G). Strong reporter activity was observed in the mesonephric ducts of both sexes throughout fetal development (Figs. 41 and 42).

After birth (P7, P21, and adult), reporter activity was exclusively detected in granulosa cells (Figs. 41K, L and 42D, E). All follicle stages were strongly stained at P7 (Fig. 42D), but β-galactosidase levels appeared to decline in primordial follicles at later stages (Fig. 42E). This pattern is more exclusive than that observed by Tanwar and McFarlane (2011), who observed pSMAD1/5/8 expression in the oocytes, granulosa, and theca cells of growing follicles as well as the ovarian surface epithelium (Tanwar et al., 2011). Testes were not examined for BRE reporter activity at postnatal stages.
Figure 41. Time course of BRE-lacZ expression in male and female fetal gonads and prepubertal ovaries as determined by X-gal staining. Strong reporter activity was observed in the mesonephric ducts of both sexes throughout fetal development (arrow in top left panel). (A, B) At E11.75, X-gal activity was detected in the gonads of both sexes. (C, D) By E12.5, X-gal staining was stronger in the ovary than the testis. This pattern continued until birth (E-J). X-gal staining in testes became restricted to the interior vasculature and coelomic vessel (arrowhead in (J)). The strong X-gal staining observed in P7 ovaries (K) became restricted to follicles by P21 (L). Testes were not examined for BRE reporter activity at postnatal stages. Bright-field images were all taken at the same magnification. nd, not done.
Figure 41: Time course of *BRE-lacZ* expression in male and female fetal gonads and prepubertal ovaries as determined by X-gal staining
Figure 42. Time course of BRE-lacZ expression in fetal and prepubertal ovaries as determined by immunostaining. Ovaries were immunostained with antibodies against β-galactosidase (green), GATA4 or FOXL2 (to label all or a subset of somatic cells, blue), PECAM (to label germ cells and endothelial cells, magenta) or E-cadherin (to label germ cells, magenta). (A, B) At E11.75 and E12.5, β-galactosidase immunostaining was localized to endothelial cells (arrow in B') and scattered GATA4-positive somatic cells located near the top and middle of the gonad. No reporter activity was observed in the outermost coelomic domain or in the germ cells. (C) By E14.5, β-galactosidase was excluded from the coelomic surface, germ cells, stromal/interstitial cells (arrowhead in C'), and endothelial cells, but it was strongly expressed in the FOXL2-positive cells that make up the rest of the somatic compartment. (D) By one week after birth (P7), reporter activity was exclusively detected in granulosa cells in all stages of folliculogenesis and entirely absent in the interstitium. (E) As the ovary matured (P21 and adult (data not shown)), β-galactosidase staining remained strong in growing follicles but declined in primordial follicles and was absent in corpora lutea (not shown). The images in (A-E) were taken at 80×; (A'-D') were taken at 400×; and (E') was taken at 200×. Scale bars represent 50 μm.
Figure 42: Time course of **BRE-lacZ** expression in fetal and prepubertal gonads as determined by immunostaining
8.3.2 BMP signaling in $Wnt4$ mutant and $Foxl2$ hypomorphic ovaries

$Bmp2$ expression is absent in XX $Wnt4^{-/-}$ gonads at E12.5 (Yao et al., 2004). Because it was unclear whether the $BRE$-$lacZ$ reporter was responding to BMP2 or another BMP ligand, we assessed BRE reporter activity in $Wnt4^{-/-}$ mutant ovaries at E13.5. In agreement with the previous findings, reporter activity was almost completely abolished in the absence of $Wnt4$ (Fig. 43A-F). Residual staining was observed in a few scattered somatic cells as well as the ectopic coelomic vessel that forms in $Wnt4$ mutants (Fig. 43B, D, F), similar to the pattern observed in the testis. This result does not necessarily indicate that the reporter is responding to signaling from BMP2, but, if not, the stimulatory ligand must also be downregulated at this stage in the $Wnt4$ mutant.

By E16.5, reporter activity was recovered in FOXL2-expressing cells at the anterior end of the $Wnt4^{-/-}$ mutant ovary, where germ cells are absent (Fig. 43G-J). These cells also express AMH (Fig. 43K) and have been postulated to represent prematurely activated granulosa cells rather than transdifferentiating Sertoli-like cells (Maatouk et al., unpublished data). This hypothesis is consistent with the coexpression of $Foxl2$, $Amh$, and the $BRE$-$lacZ$ reporter in normal growing follicles (Fig. 42D, E; for AMH expression, see (e.g., Visser et al., 2006)). It is possible that BRE reporter activity is induced in these mutant cells by one of the ligands (e.g., $Bmp4$ or $Bmp7$) that normally regulate follicle development in the postnatal ovary rather than the Wnt-regulated ligands that activate it earlier in development.
Figure 43. Wnt4−/− ovaries transiently lose BRE reporter activity. (A-F) At E13.5, X-gal staining and β-galactosidase immunostaining were strong in controls (A, C, E) but nearly absent in Wnt4−/− ovaries (B, D, E). Residual staining was observed in a few scattered somatic cells as well as the ectopic coelomic vessel (arrowheads, stained with PECAM (blue)). No β-galactosidase colocalization with FOXL2 (magenta) was observed. (G-K) At E18.5, X-gal staining and β-galactosidase immunostaining were evenly distributed throughout control ovaries (G, I), but biased to the anterior end of Wnt4−/− ovaries (H, J), where germ cells (stained with E-cadherin, blue) were depleted (right side of ovary pictured in (J)). Here, BRE reporter activity colocalized with AMH in small cord-like structures (K). Confocal images of whole-mount immunostained ovaries were taken at 80× (C, D), 100× (I, J), 200× (K), or 400× (E, F). Bright-field images were all taken at the same magnification. Scale bars represent 50 μm. A and P indicate the anterior and posterior ends of the ovary, respectively. nd, not done.
Figure 43: Wnt4<sup>−/−</sup> ovaries transiently lose BRE reporter activity
Alternatively, recovery of BMP signaling activity in the \( Wnt4^{+/+} \) ovary might indicate that one of the previously Wnt-regulated BMP ligands (e.g., \( Bmp2 \)) is reexpressed at late stages under the transcriptional control of a different upstream regulator. The latter possibility seems plausible, as \( Bmp2 \) transcription appears to be regulated by FOXL2 as well as Wnt signaling at late fetal stages (Kashimada et al., 2011). This reasoning led us to evaluate reporter expression in \( Foxl2^{GCE/GCE} \) and \( Foxl2^{GCE/GCE}; Wnt4^{+/+} \) ovaries. Mice homozygous for the \( Foxl2^{GCE} \) allele retain a low level of FOXL2 expression (Fig. 44B) but still show the characteristic eyelid defects (Fig. 44A) reported for the null animals (Uda et al., 2004). We therefore consider \( Foxl2^{GCE/GCE} \) embryos to represent hypomorphs rather than full null mutants. By X-gal staining, expression of the \( BRE-lacZ \) reporter was considerably reduced but not abolished in XX E18.5 \( Foxl2^{GCE/GCE} \) ovaries relative to wild-type controls (Fig. 45B, D). Diffuse staining was evenly distributed throughout the ovary in this mutant, unlike the \( Wnt4^{+/+} \) ovary, in which X-gal staining was concentrated in the small cord-like structures at the anterior end of the tissue (Figs. 43H and 45C). Future work will evaluate \( BRE-lacZ \) expression in \( Foxl2^{GCE/GCE} \) samples at earlier time points, under the hypothesis proposed by Kashimada et al. (2011) that FOXL2’s regulation of \( Bmp2 \) signaling is limited to late fetal and/or postnatal stages.
Figure 44. Foxl2\textsuperscript{GCE/GCE} mutants present characteristic eyelid defects but retain a residual amount of FOXL2 protein. (A) Newborn Foxl2\textsuperscript{GCE/GCE} pups (right) exhibit the eyelid defects (arrow) seen in other Foxl2 mutant lines (compare to littermate Foxl2\textsuperscript{GCE/+} control on the left). (B) However, a low level of FOXL2 protein (green) expression was still observed in the ovary. The dotted lines indicate the ovary-mesonephros border. Confocal images of whole-mount immunostained ovaries were taken at 200× (top panels) or 400× (bottom panels).
Figure 44: Foxl2<sup>GCE/GCE</sup> mutants present characteristic eyelid defects but retain a residual amount of FOXL2 protein
Figure 45. Comparison of BRE-lacZ reporter expression in control, Wnt4, Foxl2, and Wnt4; Foxl2 double mutants. Ovaries from E18.5 control and mutant littermates were stained with X-gal. (A) No X-gal staining was detected in a Foxl2GCE/+; Wnt4+/− ovary that lacked the BRE-lacZ reporter gene. (B) Strong X-gal staining was observed in a control Foxl2GCE/+; Wnt4+/−; BRE-lacZ ovary. (C) Loss of Wnt4 resulted in localized, punctate X-gal staining at the anterior end of the Foxl2GCE/+; Wnt4−/−; BRE-lacZ ovary (see Fig. 43). (D) In the hypomorphic Foxl2 mutant (Foxl2GCE/GCE; Wnt4+/−; BRE-lacZ), X-gal staining was present but diffuse. (E) Loss of both Wnt4 and Foxl2 completely abrogated BRE reporter activity as judged by X-gal staining. Bright field images were all taken at the same magnification.
Figure 45: Comparison of BRE-lacZ reporter expression in control, Wnt4, Foxl2, and Wnt4; Foxl2 double mutants
Interestingly, X-gal staining was entirely absent in the Foxl2^{GCE/GCE}; Wnt4^{-/-} ovaries (Fig. 45E). This result suggests that the BMP ligand that is responsible for inducing BRE reporter activity in the late stage Wnt4^{-/-} mutant, be it Bmp2 or a precociously expressed ligand involved in follicle development, is transcriptionally regulated by FOXL2. Distinguishing between these possibilities will require assessing the transcript levels of various BMP ligands (2, 4, 5, 6, 7, and 15) in the Foxl2^{GCE/GCE}; Wnt4^{-/-} ovaries.

### 8.3.3 Constitutive activation of BMPR1A had no apparent effect on testis development

BMP signaling is clearly regulated by the Wnt pathway in the developing ovary, but it is still unclear whether it mediates any of the downstream effects attributed to Wnt4 signaling. Therefore, we determined the impact of constitutive BMP signaling in the testis. Both Bmpr1a and Bmpr2 are highly expressed in somatic cells of male and female gonads between E11.5 and E13.5 (Jameson et al., unpublished; Fig. 40), and BMPR1A is known to function as a type 1 receptor for BMP2 (Koenig et al., 1994). We crossed animals carrying a conditional, constitutively active allele of Bmpr1a (Rodriguez et al., 2010) to two different Cre lines. The first, Sf1-Cre, is activated in gonadal somatic cells around E11.5, while the second, Wt1^{CreER}, is also expressed in somatic cells but is tamoxifen inducible.

Surprisingly, E12.5-13.5 XY Sf1-Cre; Bmpr1a^{CA/+} animals were indistinguishable from controls in terms of testis cord structure (assessed by SOX9 and PECAM1 staining), vasculature (PECAM1), and interstitial cell specification (VCAM, 3β-HSD) (Fig. 46A and
data not shown). Likewise, XY Wt1<sup>CreER</sup>; Bmpr1a<sup>CA/+</sup> mice treated with tamoxifen at E9.5-10.5 were phenotypically normal at a gross morphological level (data not shown). Female E12.5-13.5 Sfl-Cre; Bmpr1a<sup>CA/+</sup> and Wt1<sup>CreER</sup>; Bmpr1a<sup>CA/+</sup> also did not show any obvious abnormalities (Fig. 46B). Future studies will evaluate these mutants at later fetal and early postnatal stages. We are also crossing the BRE-lacZ reporter onto these strains to determine whether BMP signaling is indeed ectopically activated in XY gonads upon Cre-mediated activation of the Bmpr1a<sup>CA</sup> allele.
Figure 46. Constitutive activation of Bmpr1a in somatic cells did not induce gross morphological changes. (A) Testes from E12.5 and E13.5 XY control Bmpr1a<sup>CA/+</sup> (left panels) and mutant Sf1-Cre; Bmpr1a<sup>CA/+</sup> (right panels) embryos were indistinguishable in terms of testis cord structure (assessed by SOX9 (magenta) and PECAM (blue) immunostaining), vascular development (PECAM), and interstitial cell specification (VCAM, green). (B) Ovaries from E12.5 and E13.5 XX control Bmpr1a<sup>CA/+</sup> (left panels) and mutant Sf1-Cre; Bmpr1a<sup>CA/+</sup> (right panels) embryos did not show any obvious differences in terms of germ cell number, vascular patterns (both assessed with PECAM staining, blue), or establishment of cell cycle arrest (p27, magenta). Confocal images of whole-mount immunostained gonads were taken at 80×. Scale bars represent 50 μm.
Figure 46: Constitutive activation of Bmpr1a in somatic cells did not induce gross morphological changes
8.4 Discussion and future directions

The above analyses strongly indicate that the BMP pathway is active in ovarian somatic cells downstream of \textit{Wnt4} and \textit{Foxl2}, but its role in fetal ovary development remains unclear. Future studies will investigate the latter using a loss-of-function model. Null mutations in \textit{Bmp2} result in early embryonic lethality (\textit{\textasciitilde}E7.5-9) (Zhang et al., 1996), precluding determination of potential gonad phenotypes. As noted above, combined mutation of the putative BMP targets \textit{Msx1} and \textit{Msx2} inhibits germ cell entry into meiosis and later results in increased germ cell death (Le Bouffant et al., 2011), but it is unclear whether these activities are endogenously regulated by BMPs. \textit{Bmpr1a}, \textit{Bmpr1b}, \textit{Smad1}, \textit{Smad5}, and \textit{Smad8} have not been conditionally deleted in the gonad during fetal stages, and the effects of \textit{Smad4} mutations have only been evaluated in the testis (Archambeault et al., 2010).

We are performing preliminary studies with dorsomorphin, a pharmacological inhibitor of BMPR1A (Yu et al., 2008). However, because this molecule has known off-target effects (Vogt et al., 2011), we will also evaluate a genetic model in which \textit{Bmpr1a} is conditionally ablated in gonadal somatic or germ cells. Though the \textit{BRE-lacZ} reporter is exclusively expressed in somatic cells, we think it important to also assess a germ cell mutant, given the previous evidence suggesting that BMP signaling promotes proliferation (Pesce et al., 2002), apoptosis (Childs et al., ), or entry into meiosis (Le
Bouffant et al., 2011) in female germ cells. It is also possible that all of these effects, if real, could be mediated indirectly by somatic cells.

Stabilization of β-catenin in the XY gonad resulted in a male-to-female phenotypic sex reversal in which SOX9 expression was downregulated, female markers were upregulated, and germ cells entered meiosis (Maatouk et al., 2008). Another line of experiments will investigate this Sfl-Cre; β-catenin<sup>3/3<sup> sex reversal model to determine whether BMP signaling is ectopically activated in XY gonads in the presence of stabilized β-catenin and might therefore mediate some aspects of the sex-reversal phenotype.

Finally, the absence of BRE reporter activity in Foxl2<sup>GCE/GCE</sup>; Wnt4<sup>+/-</sup> ovaries is consistent with the greater degree of female-to-male sex reversal previously observed in double mutant ovaries (Ottolenghi et al., 2007). It may therefore be more appropriate to consider the lack of BMP signaling as a consequence of testis differentiation rather than indicative of a requirement for dual regulation by Wnt4 and Foxl2. Given that we consider our Foxl2<sup>GCE/GCE</sup> mutants to be hypomorphs rather than null mutants (Fig. 44), distinguishing between these options will require assessing the degree of testicular differentiation in our Foxl2<sup>GCE/GCE</sup>; Wnt4<sup>+/</sup> ovaries.
Appendix B: Mouse germ cell clusters form by aggregation as well as clonal divisions

9.1 Summary

After their arrival in the fetal gonad, mammalian germ cells express E-cadherin and are found in large clusters, similar to germ cell cysts in Drosophila. In Drosophila, germ cells in cysts are connected by ring canals. Several molecular components of intercellular bridges in mammalian cells have been identified, including TEX14, a protein required for the stabilization of intercellular bridges, and several associated proteins that are components of the cytokinesis complex. This has led to the hypothesis that germ cell clusters in the mammalian gonad arise through incomplete cell divisions. We tested this hypothesis by generating chimeras between GFP-positive and GFP-negative mice. We show that germ cell clusters in the fetal gonad arise through aggregation as well as cell division. Intercellular bridges, however, are likely restricted to cells of the same genotype.

9.2 Introduction

In mammals, germ cells migrate to the gonad from the base of the allantois. From the time germ cells leave the allantois, they travel as individual cells often connected in large networks by long cytoplasmic filaments (Gomperts et al., 1994). However, when germ cells colonize the gonad, they form large clusters of eight or more cells. During this
period, germ cells are also undergoing cell divisions (Pepling et al., 1998). Whether the clusters of germ cells in the gonad arise through aggregation or cell division is unclear.

Imaging analyses suggested that filopodial connections between migrating germ cells might lead to their aggregation in the genital ridge (Gomperts et al., 1994; Bendel-Stenzel et al., 2000). When cells from the hindgut mesentery and associated mesonephroi were disaggregated and plated on STO feeder cell layers, germ cells formed clusters. Furthermore, when half of the germ cells in the cultures were labeled with rhodamine-conjugated dye, clusters were composed of labeled and unlabeled cells (Gomperts et al., 1994), compatible with the idea that clusters can arise through aggregation, rather than solely through cell division. Further investigations showed that blocking E-cadherin, which is expressed by germ cells upon arrival in the gonad (Di Carlo et al., 2000), led to a disruption in germ cell aggregation in slice cultures and in dissociated reaggregation assays (Bendel-Stenzel et al., 2000). However, it was not clear that these in vitro assays reflected aggregation properties in vivo.

Other investigators have noticed the similarity between premeiotic germ cell clusters in vertebrates and those found in invertebrates such as Drosophila. In Drosophila, germ cell cysts contain 16 cells that arise through stereotypic divisions. Divisions in the cyst are incomplete and result in ring canals that form cytoplasmic bridges between all 16 cells in the cluster (reviewed by Pepling et al., 1999). Similar intercellular bridges have been seen in electron microscopy studies of mice, rabbits, rats, hamsters, and humans.
(Fawcett 1961; Gondos 1973; Gondos et al., 1973; Pepling et al., 1998). Pepling and Spradling further analyzed these structures in the mouse ovary and showed that intercellular bridges resembling *Drosophila* ring canals are present between germ cells in the ovary between embryonic day E11.5 and E17.5. Germ cells in clusters also show synchronous cell divisions, which are characteristic of germ cell cysts in *Drosophila* and *medaka* (Pepling et al., 1999; Nakamura et al., 2010). In addition, clusters tend to be comprised of an even number of cells, consistent with the idea that clusters arise through cell division rather than random aggregation (Pepling et al., 1998).

In *Drosophila*, only one of the germ cells in the cluster becomes an oocyte, while the others develop into nurse cells that nourish the oocyte by a directional transport of mitochondria and specific mRNAs through the cytoplasmic bridges (de Cuevas et al., 1997). In mice, there is at least a 3-fold reduction in the number of germ cells in the ovary between the time that their numbers reach a maxima and the time of follicle formation just after birth (Pepling et al., 2001; McClellan et al., 2003). There is currently no clear explanation for this high level of germ cell atresia during fetal ovary development. However, the identification of endoplasmic reticulum and mitochondria within intercytoplasmic bridges between adjacent germ cells in mouse ovaries led to the idea that germ cell atresia could be explained by a selective mechanism similar to the one that operates in *Drosophila* (Pepling et al., 2001). The idea that processes underlying oocyte selection are conserved is appealing.
More recently, molecular components of intercellular bridges in mammalian cells have been identified (reviewed by Greenbaum et al., 2011). The first of these was TEX14, a protein that is required for the stabilization of intercellular bridges and essential for spermatogenesis in males (Greenbaum et al., 2006) but not oogenesis in female mice (Greenbaum et al., 2009). Using a proteomics approach on testis fractions enriched for cytoplasmic bridges, several components of the cytokinesis complex were identified and shown to colocalize with TEX14 to cytoplasmic bridges during early spermatogenesis, including three components of the midbody, the mitotic kinesin-like protein 1 (MKLP1/KIF23), RACGAP1 (MgcRacGap), and centrosomal protein 55 (CEP55), as well as several septin proteins (Greenbaum et al., 2007; Iwamori et al., 2010). These elegant studies provided clear evidence that cytoplasmic bridges can form as the result of incomplete cell divisions during germ cell development.

To investigate whether germ cell aggregates arise only through clonal divisions in mice, we produced chimeras between GFP-positive and GFP-negative embryos. Our results indicate that while fetal germ cell clusters arise through both aggregation and clonal divisions, we detected bridges only between germ cells of the same genotype.

9.3 Results

9.3.1 Determination of the sex of the two components of the chimeras

Chimeric embryos were allowed to develop to E11.5, E12.5, or E13.5 before dissection. The sex of the embryos was unknown at the time of assembly. Therefore,
chimeras could be XX↔XX, XY↔XY, or XX↔XY. To determine the XX or XY constitution of the chimeras, the fetal liver was isolated from embryos at the time of dissection. Fragments of the fetal liver were reserved for immunocytochemistry and for an unsorted control (UN). Cells in the remainder of the fetal liver were dissociated, and fluorescence activated cell sorting (FACS) was used to isolate pure populations of GFP-positive (GP) and GFP-negative (GN) cells. PCR analysis of these two populations was performed with primers that distinguish the X and Y chromosomes (Fig. 47A,D,G). This analysis determined the sex of the GFP-positive and GFP-negative components of each chimera.

To confirm the PCR results, the sex was retrospectively determined by staining chimeric tissue (limb, liver or intestine) with antibodies against histone H3 lysine 27 (H3K27), which strongly labels the inactive X chromosome in XX cells as well as heterochromatin throughout the nucleus (Rougeulle et al., 2004), and co-imaging with GFP. This assay confirmed the PCR results in all chimeras tested (Fig. 47B, E, H).
**Figure 47.** Germ cell clusters include both GFP-positive and GFP-negative cells. (A-C) Example of an XX↔XX chimera. (A) Only the X chromosome was detected by PCR analysis in unsorted (UN), GFP negative (GN), and GFP positive (GP) cells. (B) An analysis of limb tissue using antibodies against GFP (green) and H3K27 (magenta) detected the inactive X chromosome (Xi) in both GP and GN cells (arrows). (C) Image of an E11.5 gonad labeled with antibodies against GFP (green) and E-cadherin (magenta, germ cell membranes) revealed E-cadherin-mediated junctions (arrows) between GN and GP germ cells. (D-F) Example of an XX↔XY chimera. (D) Both X and Y chromosomes were detected in UN and GP cells. Only the X was detected in GN cells. (E) The Xi was only detected in GN cells (arrows). (F) In an E11.5 gonad, E-cadherin-mediated junctions (arrows) were observed between GN and GP germ cells. (G-I) Example of an XY↔XY chimera. (G) Both X and Y chromosomes were detected in UN, GP, and GN cells. (H) No Xi was detected in either GP or GN cells. (I) In an E11.5 gonad, E-cadherin-mediated junctions (arrows) were observed between GN and GP germ cells. Scale bars represent 10 µm.
Figure 47: Germ cell clusters include both GFP-positive and GFP-negative cells.
9.3.2 Germ cell clusters contain both GFP-positive and GFP-negative germ cells that share E-cadherin boundaries

Immunocytochemistry was performed on whole-mount fetal gonads in XX↔XX, XY↔XY, and XX↔XY chimeras using an antibody against E-cadherin. In most samples tested at E11.5-E13.5, clusters of germ cells contained both GFP-positive and GFP-negative germ cells (Fig. 47C, F, I), which could be detected in Z-planes of the cluster (Fig. 48A). E-cadherin-positive boundaries were detected between clustered germ cells of different XX and XY genotypes (Figs. 47C, F, I and 48A). This result was confirmed with two alternative germ cell markers, MVH and PECAM-1 (Fig. 48B, C).

9.3.3 Intercellular bridges form between germ cells of the same genotype

Control XX and XY gonads at E11.5 and E13.5 were labeled with antibodies against E-cadherin and TEX14, an element of intercellular bridges in mammalian germ cells. TEX14-positive structures were identified between E-cadherin-positive germ cells in both sexes at E11.5 and E13.5 (Fig. 49A), as reported previously (Greenbaum et al., 2009). The antibody against TEX14 was then used in combination with an antibody against E-cadherin on E12.5 and E13.5 XX↔XY, XX↔XX, and XY↔XY chimeras. The vast majority of TEX14-positive structures fell between germ cells of the same genotype in all chimeras (Fig. 49B). Out of more than 600 structures counted in an analysis of two E12.5 XX↔XY, one XX↔XX, and two XY↔XY chimeras, only six appeared to be positioned between germ cells of different genotypes, and only two of the six were convincing by
Figure 48. Germ cell clusters contain cells of different genotypes. (A) Z-sections through a cluster in an E11.5 XX↔XX chimera, showing that the presence of GFP-positive and GFP-negative germ cells in clusters is not an artifact of sectional plane. The z-sections shown are 2 µm apart. Germ cell boundaries are marked with E-cadherin (magenta). (B) A single cluster within a testis cord of an E13.5 XY↔XY chimera in which germ cells were labeled with the specific marker, MVH (magenta), showing the presence of both GFP-positive and GFP-negative cells within the cluster. (C) A single cluster within an E13.5 XX↔XX chimera in which germ cells were labeled with another marker, PECAM-1 (magenta). Scale bars represent 10 µm.
Figure 48: Germ cell clusters contain cells of different genotypes
Z-stack analysis (Fig. 49C). None of the bridges between cells of different genotypes fell in the middle of a definitive, straight border between the two germ cells, as was commonly seen between germ cells of the same genotype. This observation, together with their extremely low frequency of occurrence, suggests that these inter-genotype TEX14-positive structures may represent anomalies or imaging artifacts rather than examples of biologically relevant de novo bridge formation.

9.3.4 Synchronous cell divisions in chimeric gonads

Previous studies noted that clusters of embryonic germ cells undergo synchronized cell divisions, a process that may involve communication through intercellular bridges (Pepling et al., 1998). We predicted that if bridges were only present between clonally related germ cells and were required to coordinate the cell cycle, synchronous divisions should be restricted to cells of the same genotype, even within mixed clusters. Metaphase cells were identified by DNA condensation. Five (two E12.5 XX↔XY, two E12.5 XY↔XY, and one E11.5 XX↔XY) chimeric gonads that contained both GFP-positive and GFP-negative germ cells were evaluated in this analysis. Fourteen small clusters of two or more synchronously dividing germ cell adjacent to non-dividing germ cells of the same or opposite genotype were frequently observed in these five gonads (Fig. 50A, B), suggesting that synchronicity is restricted to clonally related cells. In fifteen other locations, a single mitotic germ cell was surrounded by non-mitotic cells of the opposite genotype (non-synchronous). In one instance, we observed four mitotic
GFP-positive cells adjacent to three mitotic GFP-negative cells (Fig. 50C). The latter case can be interpreted as either a stochastic coincidence or indicative that synchronicity of germ cell divisions does not depend on intercellular bridges.
Figure 49. Intercellular bridges in chimeric gonads. (A) TEX14-positive (magenta) intercellular bridges are present in both XX and XY germ cells (stained with E-cadherin, blue) at E11.5, but were more common in XX than XY germ cells at E13.5. (B) TEX14-labeled intercellular bridges (magenta) were present at E-cadherin-positive boundaries (blue) between germ cells of the same genotype in an E12.5 XX ↔ XY chimera. (C) Example of a putative bridge between germ cells of different genotypes in an E13.5 XX ↔ XX chimera. Such bridges were very rare and we could not rule out a connection to another germ cell of the same genotype that lay out of the confocal plane. Scale bars represent 10 µm.
Figure 49: Intercellular bridges in chimeric gonads
Figure 50. Synchronously dividing germ cells usually had the same genotype. (A) Two synchronously dividing GFP-negative germ cells labeled with E-cadherin (blue; white arrowheads) surrounded by non-dividing GFP-negative cells (yellow arrowhead) in an E12.5 XY↔XY chimera. Condensation of DNA (green) indicates that a cell is in metaphase. (A’) Same image as in (A) but with GFP overlay (magenta). (B, B’) Four synchronously dividing GFP-positive germ cells (magenta arrowheads) adjacent to non-dividing GFP-negative germ cells in an E12.5 XX↔XY chimera. Note that one GFP-positive cell in the cluster is not in metaphase (yellow arrowhead). (C, C’) A cluster from an E12.5 XY↔XY chimera showing a rare example of adjacent, synchronously dividing GFP-positive (magenta arrowheads) and GFP-negative germ cells (white arrowheads). Nuclei were labeled with DAPI (green). The scale bar represents 10 µm.
Figure 50: Synchronously dividing germ cells usually had the same genotype
9.4 Discussion

These results clearly demonstrate that germ cell clusters do not arise solely through incomplete divisions, but must also form through aggregation. Female germ cells are known to form cyst-like structures in the fetal mouse ovary and undergo synchronous cell divisions, similar to *Drosophila* germ cell cysts, which arise through incomplete divisions (Pepling et al., 1998). Our analysis determined that germ cell clusters in the fetal gonad are not uniformly clonal. Indeed, germ cell clusters that contain germ cells of different genotypes (i.e., GFP-positive and GFP-negative) cannot have arisen by cell division. The mechanisms that promote germ cell aggregation as germ cells arrive in the fetal mouse gonad are presently unknown, although they likely involve E-cadherin.

Intercellular bridges within the mixed germ cell clusters were frequently observed between cells of the same genotype and only rarely found between cells of different genotypes (2/600 bridges), suggesting that if bridges actually occur between non-clonal cells, they are unlikely to have biological relevance within germ cell aggregates *in vivo*. This result supports previous work showing that intercellular bridges between mammalian spermatogonia contain markers of the cleavage furrow and likely form as a consequence of incomplete cell division (Burgos et al., 1955; Braun et al., 1989; Greenbaum et al., 2007; Greenbaum et al., 2011). We often observed synchronous cell divisions among germ cells of the same genotype and rarely among germ cells of different genotypes in mixed clusters. Our sample size for this analysis was small, but
the cell division patterns observed are consistent with the idea that factors passed through intercellular bridges may coordinate the cell cycle across germ cell clusters. However, we cannot exclude the possibility that germ cell divisions are coordinated by signals from their local environment.

Bridges were present between germ cells in both the testis and ovary from E11.5 to E13.5, in accord with previous observations (Pepling et al., 1998). Maintenance of these structures suggests that they could play an important role in germ cell biology. Consistent with this prediction, elimination of TEX14 caused spermatogenic arrest at the first meiotic division, resulting in male sterility (Greenbaum et al., 2006). Although loss of TEX14 also eliminated bridges at all stages of ovarian development and led to a reduced number of oocytes in the neonatal ovary, it did not affect fertility in females (Greenbaum et al., 2009). However, regulated disassembly of intercellular bridges may be involved in oocyte nest breakdown and follicle formation in the postnatal ovary, as a correlation was noted between the presence of multioocyte follicles and the persistence of intercellular bridges in pups exposed to phytoestrogens (Jefferson et al., 2006). Whether intercellular bridges play a role in coordinating germ cell development at fetal stages is currently unknown. However, clustering of germ cells in the fetal gonad does not depend on clonal cell divisions or on intercellular bridges connecting all cells in the cluster.
References


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Biography

Lindsey Anne (Barske) Mork was born on July 21st, 1982 in Putnam, Connecticut to Wendy and Jeffrey Barske, as the younger of their two daughters. Her happy childhood was spent in the rural hamlet of Thompson, Connecticut, much of it outdoors with her pony and sister Carrie. She graduated as salutatorian from the town’s public high school, Tourtellotte Memorial, in 2000, and then headed west to matriculate at Pomona College in Claremont, California. Here, she majored in biology, spent a semester at James Cook University in Townsville, Australia, and worked as a field assistant for two summers at the Toolik Lake field station in Alaska. Upon graduating from Pomona with a bachelor’s of arts in 2004, Lindsey was fortuitously introduced to the brother of a very good friend, who kindly didn’t make a fuss when Nicolai and Lindsey fell for each other and eventually got hitched six years later. Lindsey and Nicolai lived in Century City, California, from 2004 to 2006, while she gained lab experience in an epigenetics cancer research lab at USC and then a developmental neurobiology lab at UCLA. Stirred by a fascination with evolution and embryos of all kinds, Lindsey decided to pursue graduate work in developmental biology at Duke, and, with Nicolai’s support, the pair moved back to the east coast in August 2006. Lindsey joined Blanche Capel’s lab in the spring of 2007.
Publications


