How to Make a Gonad: Cellular Mechanisms Governing Formation of the Testes and Ovaries

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Abstract

Sex determination of the gonad is an extraordinary process by which a single organ anlage is directed to form one of two different structures, a testis or an ovary. Morphogenesis of these two organs utilizes many common cellular events; differences in the timing and execution of these events must combine to generate sexually dimorphic structures. In this chapter, we review recent research on the cellular processes of gonad morphogenesis, focusing on data from mouse models. We highlight the shared cellular mechanisms in testis and ovary morphogenesis and examine the differences that enable formation of the two organs responsible for the perpetuation of all sexually reproducing species.

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Despite their anatomical and histological differences, testes and ovaries are surprisingly similar in both form and function. Both organs perform 2 essential functions in mature organisms: the first is to produce hormones, which are secreted into the blood stream and have wide-

ranging effects throughout the body. The second role of the gonad is to produce gametes. In the testis, spermatogonia are produced in the seminiferous tubules (also known as testis cords in the fetal stage), wherein a layer of Sertoli cells sequester a cluster of germ cells and secrete factors that support germ cell growth and maturation. Oocyte production in the ovary also occurs within a confined structure, the follicle. Like the seminiferous tubule, each follicle consists of an outer layer of support cells, termed granulosa cells; however, the follicle differs from the seminiferous tubule in that each follicle contains only 1 germ cell, the oocyte.

In most mammals the choice between forming a testis or an ovary is determined by the sex-determining region of the Y chromosome gene, *SRY* [Sinclair et al., 1990]. If *SRY* is expressed, from either the Y chromosome or from an exogenous transgene, testes form [Gubbay et al., 1990; Koopman et al., 1991; Eicher et al., 1995]. Individuals lacking *SRY*, such as XX genetic females or rare XY cases in which *SRY* is mutated or deleted, develop ovaries [Lovell-Badge and Robertson, 1990; Hawkins et al., 1992]. *SRY* functions as a genetic switch that directs the bipotential gonadal primordium towards testis morphogenesis. The gonadal primordia, also termed genital ridges, originate as a pair of thickened rows of coelomic epithelial cells along the inner surface of the mesonephroi (rudi-

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Fig. 1. A timeline of the major cellular events in testis and ovary morphogenesis. During mouse embryogenesis, the bipotential gonad is formed around 10.5 dpc. *Sry* is expressed from the Y chromosome beginning at 10.5 dpc in the XY gonad (top, blue) and its expression triggers the first step of testis morphogenesis (1), specification of the Sertoli cell lineage. By 12.5 dpc, the testes have compartmentalized to form defined cord structures surrounded by a basal lamina (2). This step is driven by the migration of endothelial cells into the gonad and involves the recruitment of PTM cells to surround the cords. The final stage (3) of testis development is testis cord elongation, which transforms simple

loops into a convoluted tubule structure. In the XX gonad, where *Sry* is absent (bottom, pink), events in testis morphogenesis do not happen. Specification of the granulosa cell lineage defines step 1 of ovary organogenesis (1). Female germ cells within the germ cell nest begin to enter meiosis at 13.5 dpc and arrest in the diplotene stage of meiosis I beginning at 17.5 dpc. The germ cell nests break down soon after birth (2) when granulosa cells invade the nest and surround individual oocytes to form primordial follicles. Follicle activation compartmentalizes the somatic cell environment during the first week of postnatal life (3), accompanied by the recruitment of theca cells to the primary follicle.

mentary nephric organs that will later contribute to development of the reproductive tracts). In the mouse, primordial germ cells migrate into the genital ridges of both sexes between 10.5 and 11.5 days post conception (dpc), joining the existing somatic precursor cells in a tissue with no discernable structure [Ginsburg et al., 1990]. Testis morphogenesis begins around 10.5 dpc with the expression of *Sry* and subsequent specification of the Sertoli cell lineage [Gubbay et al., 1990; Sinclair et al., 1990; Koopman et al., 1991]. From 11.5 to 12.5 dpc, the XY gonad undergoes massive growth and reorganization, ultimately resulting in formation of the testis cords, the fetal version of the seminiferous tubules (fig. 1). Each testis cord structure is composed of a central cluster of germ cells, surrounded by concentric layers of Sertoli cells, basement membrane, and peritubular myoid (PTM) cells [Skinner et al., 1985; Tung and Fritz, 1986]. Sertoli cell proliferation during the later stages of fetal testis development causes the testis cords to elongate and expand, eventually forming the seminiferous epithelium in the adult animal [Archambeault and Yao, 2010]. Germ cells in the

fetal testis are relatively quiescent; they undergo mitotic arrest as T-prospermatogonia between 13.5 and 15.5 dpc and remain in G0 until early postnatal life [McLaren, 1984].

Morphological changes in the fetal ovary are subtle compared to the fetal testis, perhaps due to the fact that somatic cell proliferation, migration, and vascularization in the ovary are either absent or occur at a lower rate than in the testis during these stages. The first somatic cell precursors specified in the ovary are pre-granulosa cells, which differentiate in response to a combination of extrinsic and intrinsic signals around 12.5 dpc [Schmidt et al., 2004; Ottolenghi et al., 2007]. Primordial follicles, the earliest stage of folliculogenesis, are formed during perinatal life when granulosa cells break down clusters of germ cells, known as the germ cell nests, into individual follicles. A single layer of granulosa cells completely surround individual germ cells and are subsequently enclosed in a thin layer of basal lamina to form primordial follicles. Theca cells are specified shortly after and localize to the outer surface of the follicle where they work to-

gether with granulosa cells to support oocyte maturation, ovulation, and hormone production. Germ cells in the fetal ovary divide by mitosis from the time they migrate into the genital ridge until approximately 13.5 dpc and then enter and arrest in meiosis I before birth [Monk and McLaren, 1981].

The seminiferous tubules of the testis and follicles of the ovary provide a confined environment without which gametogenesis will not occur. Multiple cell types must coordinate their movements and actions for each structure to form. In this review, we describe recent advances in the field of gonad morphogenesis, focusing specifically on the formation of testis cords and follicles in mice.

Making a Testis: The Step-by-Step Compartmentalization of an Amorphous Primordium into Cord Structures

The XY gonad transforms itself from a structure-less mass of cells into a defined organ within a very short period of time in mice. This incredible transformation occurs via 3 major steps (fig. 1): (1) commitment and expansion of the Sertoli cell lineage; (2) compartmentalization of the presumptive testis primordium into cords, and (3) elongation of the testis cords to form the seminiferous tubules. Each step is guided by a combination of cell autonomous and intercellular signals.

Step 1: Sertoli Lineage Commitment and the First Wave of Proliferation

Sertoli cells coordinate nearly all aspects of testis morphogenesis and as such, specification of the Sertoli cell lineage is absolutely essential for testis formation [Skinner et al., 1985; Brennan and Capel, 2004]. Sertoli cells arise from 1 of 2 possible sources: steroidogenic factor 1 or SF1-positive cells pre-existing in the bipotential gonad or progenitor cells that derive from the coelomic epithelium. Beginning around 10.5 dpc, a subpopulation of SF1-positive cells in the gonad begins to express *Sry* and, subsequently, commits to the Sertoli lineage [Gubbay et al., 1990; Koopman et al., 1990; Lovell-Badge and Robertson, 1990; Hacker et al., 1995]. Sertoli cells also differentiate from a population of progenitor cells located in the coelomic epithelium, a single layer of cells that cover the entire coelomic cavity, including the genital ridges [Karl and Capel, 1998]. Lineage tracing studies in mice have

revealed that coelomic epithelial cells enter the XY gonad between 11.2 and 12.5 dpc and give rise to both Sertoli and interstitial cell types. Remarkably, the coelomic epithelium is only competent to specify Sertoli cells during a 2-hour window of time, from 11.2 to 11.4 dpc. Beyond this time, cells of the coelomic epithelium differentiate exclusively into interstitial cells [Karl and Capel, 1998; Schmahl et al., 2000].

Sertoli cell differentiation is controlled by multiple molecular factors, all of which lie downstream of the Y chromosome-linked gene *SRY*. In the mouse gonad, *Sry* is only expressed from 10.5 to 12.5 dpc and is restricted to a single cell type, the Sertoli cell [Albrecht and Eicher, 2001]. The brief period of *Sry* expression suggests that *SRY* is important for initiation of Sertoli cell differentiation rather than maintenance of the Sertoli cell lineage [Koopman et al., 1990; Hacker et al., 1995; Bullejos and Koopman, 2001]. Factors controlling *Sry* activity have been described in other reviews in this issue and elsewhere [Bogani et al., 2009; Wainwright and Wilhelm, 2010] and will not be discussed further here. The earliest known target of *SRY* is the transcription factor *SOX9* (Sox9). *SRY* activates the production of *SOX9* in Sertoli cell precursors [Kent et al., 1996; Sekido and Lovell-Badge, 2008] and *SOX9* in turn upregulates additional genes involved in Sertoli cell differentiation, including fibroblast growth factor 9 (*Fgf9*) and prostaglandin D synthase (*Ptgds*). *Ptgds* encodes an enzyme that catalyzes the conversion of prostaglandin H₂ (PGH₂) to prostaglandin D₂ (PGD₂) [Adams and McLaren, 2002; Wilhelm et al., 2005; Kim et al., 2006]. *SOX9*, unlike *SRY*, is expressed throughout fetal life and persists in adult testes [Kent et al., 1996]. There are at least 2 different mechanisms by which *SOX9* levels are maintained in the fetal testis (fig. 2): the first is an autoregulatory feedback loop in which *SOX9* activates its own transcription [Sekido and Lovell-Badge, 2008] and the second is a positive feedback loop in which *SOX9* downstream targets such as *FGF9* and *PGD2* activate the expression of *Sox9* and consequently set up their own positive feedback loops [Kim et al., 2006; Moniot et al., 2009]. Mice lacking *Fgf9* are initially capable of upregulating *Sox9* expression, but cannot maintain *SOX9* levels and ultimately develop defects in Sertoli cell differentiation and testis cord formation [Kim et al., 2006]. At least four receptors for FGFs exist in mammals, but *FGFR2* seems to be the sole receptor for *FGF9* in Sertoli cells [Kim et al., 2006; Bagheri-Fam et al., 2008]. Deletion of *Fgfr2* phenocopies the loss of *Fgf9*, with mutant XY individuals undergoing male-to-female sex reversal. The phenotype of mice lacking the *PGD2*-produc-

Fig. 2. Mechanisms of Sertoli cell expansion. Two waves of Sertoli cell expansion occur during testis morphogenesis. **A** Sertoli cell progenitors undergo the first wave of expansion in response to an autoregulatory feedback loop initiated by SRY. SRY induces the expression of *Sox9*, which promotes the production of FGF9 and PGD2. These 2 factors activate their respective receptors (FGFR2 and DP, respectively) on neighboring pre-Sertoli cells to form positive feedback loops that maintain SOX9 expression and stimulate Sertoli cell proliferation. **B** The second wave of Sertoli cell expansion in the mouse testis occurs after 15.5 dpc. This expansion is triggered by Leydig cell-derived activin A, which induces Sertoli cell proliferation via SMAD4.

ing enzyme L-PGDS is also similar; *Sox9* mRNA levels are reduced by approximately half compared to controls and Sertoli cell differentiation and testis cord formation are stunted [Moniot et al., 2009]. *Fgf9* expression is not affected in testes lacking *L-Pgds*, nor is *L-Pgds* expression altered in *Fgf9/Fgfr2* null testes, indicating that these 2 loops work independently to maintain *Sox9* expression and that these 2 pathways by themselves are not sufficient to maintain testis morphogenesis [Bagheri-Fam et al., 2008; Moniot et al., 2009].

The earliest physical change exhibited by the XY gonad is an increase in somatic cell proliferation at 11.25 dpc [Schmahl et al., 2000; Schmahl and Capel, 2003]. Prior to this time, proliferation is slow and does not differ in XY versus XX gonads. The XY gonad responds to this burst of cell proliferation by rapidly increasing in size, roughly doubling in width every 24 h between 11.5 and 13.5 dpc [Nel-Themaat et al., 2009]. Schmahl and others labeled proliferating cells in the bipotential gonad with

bromodeoxyuridine (BrdU) and characterized the behavior of these cells in normal gonads. They found that a male-specific increase in coelomic epithelial cell proliferation occurs in 2 distinct stages, with pre-Sertoli (*Sry*⁺) cells proliferating during the first stage but not the second [Karl and Capel, 1998; Schmahl et al., 2000]. The spatial restriction of cell proliferation within the gonad also varied by stage; proliferation is initially restricted to cells located at or near the coelomic epithelium and later expands to all regions of the gonad, except for the germ cells [Schmahl et al., 2000].

Proliferation is important not only for growth of the fetal testis, but also for maintenance of the Sertoli cell lineage. Fetal testes treated with the proliferation inhibitors 5-fluorouracil (5-FU) or methotrexate (MTX) during a critical 8-hour window from 10.8 to 11.2 dpc have reduced numbers of Sertoli cells and fail to form testis cords [Schmahl and Capel, 2003]. Although reduced in number, SOX9-positive cells are present in 5-FU/MTX treated testes, indicating that proliferation is important for maintenance of committed Sertoli cell populations but does not appear to affect the initial process of Sertoli lineage specification. One factor known to be involved in both Sertoli cell differentiation and proliferation is FGF9 (fig. 2). XY gonads lacking *Fgf9* are smaller than control organs, have reduced numbers of SOX9-positive Sertoli cells, and fail to develop testis cord structures [Colvin et al., 2001; Schmahl et al., 2004]. Interestingly, FGF9 and its receptors are also present in XX gonads, yet ovarian growth is not altered following deletion of *Fgf9* [Schmahl et al., 2004]. The male-specific effect of FGF9 on gonad growth is potentially due to the sexually dimorphic nuclear expression pattern of its primary gonad receptor FGFR2, which is present in the nucleus of XY but not in XX gonads at 11.0 dpc [Schmahl et al., 2004].

The molecular mechanisms controlling the first wave of proliferation in Sertoli cell precursors are summarized in figure 2A. *Sry* is expressed in the XY gonad from 10.5 to 12.5 dpc. It induces *Sox9* expression in a cell-autonomous manner and thereby sets up an autoregulatory feedback loop in which SOX9 maintains its own expression. SOX9 also promotes the production of FGF9 and PGD2, which activate their respective receptors in a paracrine, and possibly an autocrine, manner. FGF9 and PGD2 form a positive-feedback loop with SOX9 in which they upregulate *Sox9* expression and ultimately activate their own production. Together, these loops stimulate Sertoli cell differentiation and proliferation, 2 events that are absolutely essential for testis formation.

Step 2: Compartmentalization and Testis Cord Formation

Following specification of the Sertoli cell lineage, the XY gonad begins to partition itself into the 2 compartments necessary for normal testis function: the gamete-producing testis cords and the hormone-producing interstitium. There are 3 critical stages in testis cord formation. (1) First, cells from the coelomic epithelium migrate into the testis to establish the interstitial cell population of the testis. (2) Next, the primitive vasculature underlying the mesonephros breaks down, migrates into the gonad, and reassembles to form the elaborate testis-specific vasculature from which the cords will find their shape. (3) Finally, the cords are enclosed in a layer of basal membrane to create the enclosed environment necessary for sperm production.

(1) Cell Migration from the Coelomic Epithelium and Mesonephros into the Fetal Testis

Cells from 2 neighboring structures, the coelomic epithelium and the mesonephros, are known to migrate into the fetal testis. Coelomic epithelial cells are the first cell type to migrate into the gonad. They begin to migrate into gonads of both sexes at 11.2 dpc, coincident with the first wave of pre-Sertoli proliferation in the testis [Karl and Capel, 1998]. In the XY gonad, these coelomic epithelial cells give rise to multiple cell lineages including Sertoli cells and interstitial cells. The developmental fate of these coelomic epithelial cells becomes restricted as development proceeds such that only interstitial cells are specified after 11.4 dpc. Coelomic epithelial cells also migrate into the XX gonads during this period, however, no obvious fate restriction was observed in these cells [Karl and Capel, 1998].

From 11.5 to 16.5 dpc, a second population of cells from the neighboring mesonephros migrates into the XY but not the XX gonad [Martineau et al., 1997]. Mesonephric cell migration is absolutely essential for testis cord formation. XY gonads cultured separately from their mesonephros or with a physical barrier between gonad and mesonephros cannot form testis cord structures [Buehr et al., 1993; Tilmann and Capel, 1999]. Unlike the coelomic epithelial cells that migrate into the gonad, mesonephric cells are never capable of becoming Sertoli cells. Initial studies suggested that at least 3 different cell types (PTM cells, steroidogenic cells, and cells from the vasculature) are derived from migratory cells from the mesonephros [Wartenberg, 1978; Buehr et al., 1993; Martineau et al., 1997]; however, more recent findings have demon-

strated that the mesonephric cells that migrate into the testis are exclusively endothelial [Cool et al., 2008; Combes et al., 2009].

The central role of cell migration in testis cord formation is proven by a series of gonad 'sandwich culture' experiments. As the name implies, sandwich cultures consist of different combinations of labeled and unlabeled XX or XY gonads, which are surgically separated from their respective mesonephroi and reassembled as chimeric gonad-mesonephros sandwiches in culture. Cell migration can be induced in the XX gonad when it is sandwiched between a mesonephros (either XX or XY) and a piece of an XY gonad [Martineau et al., 1997]. Furthermore, XX gonads in these sandwiches reorganize into testis cord-like structures containing basal lamina deposits and SOX9-positive Sertoli cells [Tilmann and Capel, 1999]. These findings prove that the fetal ovary is competent to undergo the male-specific processes of migration and cord formation, but simply lacks the signals necessary to do so. Sertoli cells produce a number of chemotactic signals that could induce mesonephric cell migration and potentially mediate cord formation in XX gonad sandwich cultures, including platelet-derived growth factor alpha (PDGF α), nerve growth factor (NGF), anti-Müllerian hormone (AMH), and the TGF- β family members activin A and B [McLaren, 1991; Buehr et al., 1993; Cupp et al., 2000; Brennan et al., 2003; Ross et al., 2003; Yao et al., 2006].

Together, these findings indicate that cell migration into the gonad is a necessary process in testis cord formation. As with many other processes in gonad morphogenesis, there is a time-specific requirement for cell migration in testis cord formation and the testis cords will not form if mesonephric cell migration occurs after a critical window of development [Tilmann and Capel, 1999].

(2) Vascularization Defines the Boundary between Testis Cords

The mesonephric cell population that migrates into the fetal testis is composed exclusively of endothelial cells [Cool et al., 2008; Combes et al., 2009]. These endothelial cells contribute to formation of the vascular network of the testis, which is critical both for formation of the testis cords during fetal development and for circulation of testosterone throughout the body during adulthood. In the XY gonad, vessel formation is controlled by *Sry* and begins around 12.0 dpc. Prior to this time, the genital ridges of both sexes contain a primitive vascular system composed of small branches extending from the mesonephric vascular plexus (MVP) into the gonad (fig. 3A). Multiple mouse models exhibit simultaneous defects in vascular-

Fig. 3. Mechanisms of endothelial cell migration and testis cord formation. **A** At 11.5 dpc, there is minimal vasculature in the testis, but a prominent vessel structure, the mesonephric vascular plexus (MVP), is present in the neighboring mesonephros. 12 h later, the MVP breaks down and individual endothelial cells are observed to migrate into the testis, towards the coelomic domain. By 12.5 dpc, the coelomic vessel is clearly visible at the coelomic domain of the testis and testis cords have formed along the tracks followed by the migrating endothelial cells. **B** At the molecular

level, SRY and its downstream effector SOX9 control sexually dimorphic vascularization of the gonad. In the XY gonad (right) SRY/SOX9 suppress *Wnt4* and its downstream target *Fst*. In the absence of WNT4/FST, activin B becomes available and along with other possible factors, including VEGF, promotes vascularization of the testis. When SRY/SOX9 are absent, such as in the ovary (left), the inhibition on *Wnt4* and *Fst* is relieved. WNT4/FST therefore repress the activity of activin B and subsequently prevent the testis pattern of vascularization.

ization and cord formation, adding further support to the hypothesis that these processes are essential to testis morphogenesis [Albrecht et al., 2000; Brennan et al., 2003; Yao et al., 2006].

The fetal testes utilize a unique mechanism of endothelial cell recycling to vascularize the gonad. Figure 3A illustrates the findings of Coveney et al. [2008], who utilized time-lapse confocal microscopy to follow individual cells in cultured gonads during the critical period of vascular remodeling, 11.5 to 13.5 dpc, in the mouse. Their

results show that vascularization of the XY gonad is a dynamic process, resulting from the breakdown and remodeling of the rudimentary MVP. Individual cells from the MVP migrate to the coelomic domain at the distal edge of the testis and re-aggregate to form the coelomic vessel, the most prominent vessel of the testis, and associated minor vessels. These processes do not occur in XX gonads.

Interestingly, all endothelial cell migration in the XY gonad occurs along a small number of set paths, which appear to be determined spontaneously by the first pio-

neer cells that cross the gonad [Coveney et al., 2008]. What are the signals responsible for attracting this first group of pioneer cells to the coelomic surface of the gonad? One possible candidate is vascular endothelial growth factor (VEGF), which is expressed specifically in the undifferentiated mesenchyme of the testis interstitium. XY gonads cultured with a VEGF inhibitor failed to form testis cords and exhibited reduced interstitial cell proliferation and vascular development [Cool et al., 2011]. The receptors for VEGF (FLK-1 and NRP1) are localized to the endothelial cells of the gonad, suggesting that VEGF signals from the testis interstitium to control endothelial migration and cord formation. It should be noted that VEGF is also expressed in the fetal ovary, and therefore, VEGF may not be the migration-inducing factor but rather acts downstream of endothelial migration to control the assembly of endothelial cells into vascular networks.

Studies of the ovary-specific signaling molecule WNT4 provide more definitive genetic evidence for the molecular mechanisms controlling endothelial cell migration in the developing testis. *Wnt4* becomes an ovary-specific gene after 11.5 dpc [Vainio et al., 1999]. Together with its downstream target follistatin (*Fst*), WNT4 represses endothelial cell migration and subsequent vascularization of the fetal ovary, based on the observation that loss of either *Wnt4* or *Fst* results in ectopic formation of the testis-specific coelomic vessel in the fetal ovary [Vainio et al., 1999; Yao et al., 2004]. The WNT4/FST pathway in the ovary suppresses testis vasculature formation by antagonizing activin B, a member of the transforming growth factor (TGF) β superfamily. Under normal circumstances, activin B expression is low in the fetal ovary and high in the fetal testis cords [Yao et al., 2006]. In the absence of *Wnt4*, ovarian expression of activin B is significantly elevated. FST, on the other hand, inhibits activin B action by preventing it from binding its receptor. The ovary vasculature defects of *Wnt4*^{-/-} and *Fst*^{-/-} animals are corrected by co-deletion of activin B, thereby confirming that ectopic formation of the testis vasculature is induced by activin B [Yao et al., 2006; Liu et al., 2010a].

Whether activin B is involved in endothelial cell migration and vasculature formation in the fetal testis remains to be determined. The coelomic vessel in the activin B knockout testis is significantly smaller in diameter than the wild type control, suggesting that other factors could work synergistically with activin B. This hypothesis is supported by the findings that activin B and other TGF β proteins, such as AMH, a Sertoli cell-specific

protein in the fetal testis, and bone morphogenetic protein (BMP), are able to induce ectopic formation of testis vasculature in the wild type ovary [Ross et al., 2003]. A working model for the establishment of testis-specific vasculature is proposed in figure 3B. At 12.5 dpc, the SRY/SOX9 signaling cascade inhibits the WNT4/FST pathway, allowing activin B, VEGF, and other unidentified factors to act. These factors together facilitate endothelial migration and formation of the testis-specific vasculature. At the same time in the fetal ovary, the absence of SRY/SOX9 leads to upregulation of WNT4 and FST. The WNT4/FST pathway antagonizes activin B, thereby preventing coelomic vessel formation.

(3) Peritubular Myoid Cell Recruitment and Deposition of the Basal Lamina That Delineates the Testis Cords

The final stage of testis compartmentalization is the formation of the basal lamina to establish a physical barrier around the testis cords. In vitro organ culture experiments demonstrated that suspensions of dispersed neonate testicular cells, enriched for Sertoli cells, are capable of self-assembling into cord-like structures [Hadley et al., 1985]. These findings indicate that cord formation is a cell-intrinsic property of Sertoli cells; however, subsequent studies have revealed that at least 1 other cell type (the PTM cell) is required for the formation of functional testis cords. The source of PTM cells is not known. Migratory cells from the mesonephros do not contribute to the PTM cell population, suggesting that PTM cells probably originate from an unknown somatic cell population in the gonad [Cool et al., 2008; Combes et al., 2009]. PTM cell differentiation is thought to be triggered by factors secreted by Sertoli cells, such as the morphogen Desert hedgehog (DHH) [Clark et al., 2000; Yao and Capel, 2002]. This hypothesis is supported by data from *Dhh* null mice, which develop abnormal PTM cells and malformed testis cords [Pierucci-Alves et al., 2001]. Following formation of the testis cords, PTM cells assemble around the outer border of the cords and interact with Sertoli cells to produce the extracellular matrix (ECM) proteins laminin, fibronectin, and collagen [Hadley et al., 1985; Skinner et al., 1985]. These ECM proteins make up the basal lamina that delineates the cords and provides an enclosed environment for sperm production.

Two predominant hypotheses currently exist to explain how Sertoli and germ cells physically reorganize to form the testis cords (fig. 3). The first theory proposes that endothelial cell migration during testis vascularization forms paths in the testis interstitium that will subse-

quently be used to partition the amorphous primordial into cord structures [Coveney et al., 2008]. The vasculature of the XY gonad is formed by endothelial cells that migrate into the gonad along approximately 10 distinct avascular domains. The tracks left behind by migrating endothelial cells correspond to domains located between regions where de novo condensation of the testis cords will later occur, suggesting that endothelial cell migration sets the pattern for future testis cord organization. A second hypothesis explaining testis cord morphogenesis is that interstitial cells surrounding the presumptive Sertoli germ cell mass exert 'external forces' that mechanically constrict the developing cords and that these forces dictate cord organization. Nel-Themaat and others used 4-dimensional imaging to analyze Sertoli cell morphology and behavior in the intact testis of transgenic reporter embryos. They discovered that testis cord formation is initially highly dynamic; Sertoli cells are readily exchanged between neighboring cords [Nel-Themaat et al., 2009]. The plasticity of these early cords allows branches to spontaneously form and/or fuse to efficiently fill the inner volume of the testis cords.

Both hypotheses suggest that testis cord formation is driven by cell morphogenesis events outside of the presumptive testis cords, rather than by behaviors intrinsic to the Sertoli cells themselves. Sertoli cells are however necessary for the final stage of testis cord formation, deposition of the basal lamina. They produce DHH (and potentially other factors) that stimulate PTM cell differentiation [Clark et al., 2000; Pierucci-Alves et al., 2001; Yao and Capel, 2002] and work together with PTM cells to deposit the basal lamina around the cords [Skinner et al., 1985]. Together, these data form a model for testis cord formation in which endothelial cell migration defines the initial location and structure of the cords. External forces subsequently reorganize those primitive structures to form efficiently packaged testis cords. Finally, Sertoli cells coordinate with PTM cells to deposit a basal lamina around the cords to delineate the cord structures and create an enclosed environment in which spermatogenesis will later occur.

Step 3: Second Wave of Sertoli Cell Expansion and Testis Cord Elongation

Late in fetal development, the testis cords undergo a poorly understood process of elongation and expansion, leading to formation of the adult seminiferous epithelium structure. The fact that Sertoli cells are intimately in-

involved in both initial Sertoli cell proliferation and testis cord formation programs has led many to assume that the late embryonic processes of testis cord elongation and coiling are also under the control of Sertoli cell-intrinsic programs. Recent findings have suggested that fetal Leydig cells, not Sertoli cells, control testis cord expansion during late fetal development. Inactivation of the TGF β family member activin A specifically in fetal Leydig cells does not affect initial testis cord formation; however, testis cords fail to expand and elongate after 15.5 dpc [Archambeault and Yao, 2010]. The testis cords of activin A mutant embryos exhibit abnormal coiling and decreased Sertoli cell proliferation, indicating that fetal Leydig cell-derived activin A is essential for late testis cord expansion. These observations uncovered a unique role for fetal Leydig cells as regulators of Sertoli cell proliferation in the expanding testis cords (fig. 2B).

Making an Ovary: Assembly of Ovarian Follicles

Ovary formation was for many years considered to be the default gonad development pathway, followed only in the absence of SRY expression. We now know that a number of essential ovary-specific factors exist (β -catenin, follistatin, FOXL2, R-spondin, and WNT4) without which ovary development cannot take place (see other reviews in this issue and Liu et al. [2010a]). Oocyte maturation occurs within the ovarian follicles, each composed of a single oocyte surrounded by granulosa and theca cells. In the mouse, ovarian follicles start to form perinatally. At birth, the mouse ovary is composed of basal membrane-bound clusters of oocytes and epithelial cells, known as germ cell nests, which are surrounded by mesenchymal cells. During the first week of life, the germ cell nests break down and are surrounded by somatic cells to form primordial follicles [Hirshfield, 1991]. Three critical cellular events shape ovarian follicle development: (1) specification of the granulosa cell lineage; (2) follicle formation, and (3) compartmentalization of somatic cell environment (see fig. 1).

Step 1: Specification of the Granulosa Cells

Granulosa cells are the female equivalent of the Sertoli cells; they enclose germ cells and secrete factors necessary for oocyte growth and maturation. Morphological and histological data point to 3 possible sources from which granulosa cell precursors could originate: the

ovarian surface epithelium, mesonephric cells from the adjacent rete ovarii, and the existing mesenchymal cells of the genital ridge. Given their functional similarities, it has been hypothesized that Sertoli and granulosa cells share a common precursor and that, like Sertoli cells, granulosa cells originate from the surface epithelium of the gonad. Coelomic epithelial cells are observed to migrate into the ovary; however, the fate of these cells is undetermined due to a lack of appropriate markers [Karl and Capel, 1998; Sawyer et al., 2002]. Using fluorescent dye to mark the coelomic epithelium, Mork and colleagues discovered that many coelomic epithelial cells ingress to ovarian cortex and give rise to FOXL2-positive granulosa cells [Mork et al., 2012]. A second potential source for granulosa cells is the neighboring mesonephric cells of the rete ovarii. Previous analyses have shown that this migration does not occur between 11.5 and 16.5 dpc [Byskov and Lintern-Moore, 1973; Byskov, 1975, 1978; Martineau et al., 1997]; however, the possibility remains that mesonephric migration into the ovary occurs prior to or after this time. The third possible origin for granulosa cells is that they are present in the genital ridge prior to the time of sex determination. Albrecht and Eicher [2001] generated mice carrying a transgene in which EGFP is expressed by the endogenous *Sry* promoter and found that Sertoli and granulosa cells are derived from a common precursor population. They also discovered that granulosa cell precursors are present in the gonadal ridge prior to the start of coelomic cell migration [Albrecht and Eicher, 2001]. A recent study by Mork et al. [2012] confirms these findings and furthermore uncovers unique temporal differences in granulosa cell specification in which granulosa cells of perinatal follicles are specified via different mechanisms than those utilized by granulosa cells in adult follicles. Contribution of more than one of these sources to the granulosa cell lineage cannot be excluded.

One essential factor for granulosa cell differentiation is the forkhead transcription factor FOXL2 [reviewed by Pisarska et al., 2011]. *Foxl2* is one of the earliest known markers of ovarian differentiation, present in pre-granulosa cells of the mouse ovary as early as 12.5 dpc [Schmidt et al., 2004; Ottolenghi et al., 2007]. Female mice lacking *Foxl2* fail to undergo follicle maturation; their follicles arrest between the primordial and primary stages and subsequently degenerate [Schmidt et al., 2004; Uda et al., 2004]. FOXL2 suppresses genes involved in terminal granulosa cell differentiation, such as the steroidogenic acute regulatory (*Star*) gene [Pollack et al., 1997] and *Cyp19a1* [Baron et al., 2004], and is thought to prevent

premature depletion of ovarian follicles by blocking the differentiation and/or proliferation of granulosa cells in small and medium follicles. *Foxl2* is also essential for maintenance of the granulosa cell lineage throughout adult ovary development. Remarkably, loss of *Foxl2* in the adult mouse results in a phenotype of ovary-to-testis sex reversal. The testis-specific genes *Sox9* and *Dhh* are up-regulated following deletion of *Foxl2*, resulting in the cell-autonomous reprogramming of granulosa cells into Sertoli-like cells [Uhlenhaut et al., 2009]. *Foxl2* null ovaries also developed Leydig-like cells capable of secreting testosterone at levels comparable to normal male littermates. Genetic analyses suggest that FOXL2 and the estrogen receptors (ER) α/β cooperate to maintain the granulosa cell lineage in adult animals [Uhlenhaut et al., 2009]. XX mice lacking both ER α and ER β or the estrogen producing enzyme aromatase undergo a similar adult sex reversal phenotype to that observed in *Foxl2* mutant animals [Couse et al., 1999; Britt et al., 2001; Uhlenhaut et al., 2009]. Removing 1 copy of *Foxl2* from an *Esr1* (ER α) null background results in the appearance of Sertoli-like cells, which are not present in ovaries lacking only *Esr1* [Uhlenhaut et al., 2009]. Finally, FOXL2 and ER α were observed to synergistically repress *Sox9* expression in vitro, suggesting that these 2 proteins work together to repress *Sox9* expression in adult females, thereby preventing Sertoli cell differentiation and maintaining the granulosa cell lineage [Uhlenhaut et al., 2009].

In addition to FOXL2/ER, activators of the β -catenin pathway including WNT4 and R-spondin (RSPO1) are essential for commitment of the granulosa cell lineage in the fetal ovary. *Rspo1* and *Wnt4* are expressed in the gonads of both sexes prior to the time of sex determination, and they are expressed in an ovary-specific pattern after that time [Parma et al., 2006; Chassot et al., 2008; Tomizuka et al., 2008]. Genetic analyses have revealed that RSPO1 stimulates the expression of *Wnt4*, and that RSPO1 and WNT4 work synergistically to activate β -catenin, the intracellular regulator of the WNT pathway [Yao et al., 2004; Chassot et al., 2008; Manuylov et al., 2008; Liu et al., 2010b]. XX gonads lacking *Rspo1* and *Wnt4* initially develop into ovaries but acquire testis characteristics, such as Sertoli cells and testis cords, after birth [Chassot et al., 2008; Tomizuka et al., 2008]. The RSPO1/WNT4 pathway appears to function independently of FOXL2, as demonstrated by the fact that *Rspo1/Wnt4* expression is not affected when *Foxl2* is deleted, and vice versa [Ottolenghi et al., 2007; Chassot et al., 2008]; however, these pathways also converge to regulate *Sox9* expression. Simultaneous loss of both *Foxl2* and

Fig. 4. Establishment of the theca cell lineage. Theca cells are recruited to primordial follicles during the first week of postnatal life. The origins of theca progenitor cells are currently not known; 2 possible sources, the mesonephros and the ovarian stroma, are labeled with question marks. Following primordial follicle activation, the oocyte and/or somatic cells of the follicle produce factors that promote the differentiation and migration of theca progenitor cells. Multiple stages of follicle development are illustrated, ranging from primordial follicle (A) to primary follicle (B).

Wnt4 causes a significant increase in *Sox9* expression, resulting in female-to-male sex reversal [Ottolenghi et al., 2007]. These findings combine to form a model where, in the absence of *Sry*, *FOXL2*, *RSPO1*, and *WNT4* coordinately prevent *Sox9* expression, and in doing so, suppress testis differentiation and allow ovarian structures to form [for review, see Liu et al., 2010a].

Step 2: Formation of the Follicles

The ovarian follicle is the functional unit of the female reproductive system, consisting of a single oocyte surrounded by somatic granulosa and theca cells. The precursors to mature follicles, termed primordial follicles, are formed around the time of birth when pre-granulosa cells invade clusters of germ cells, termed germ cell nests, and start to break down the nest structure (fig. 4). Individual oocytes are then surrounded by pre-granulosa cells to complete primordial follicle formation [reviewed in Edson et al., 2009]. In contrast to the formation of testis cords, ovarian follicle development is critically dependent on the presence of germ cells [Merchant-Larios and Centeno, 1981; Behringer et al., 1990]. Primordial follicles will not form if germ cells are absent and the loss of even a few germ cells can block granulosa cell differentiation, resulting in the formation of fibrous streak ovaries [Merchant, 1975]. Germ cell loss in the ovary after follicle formation causes granulosa cells to transdifferentiate into Sertoli cells [Behringer et al., 1990; Hashimoto et al., 1990; McLaren, 1991; Taketo et al., 1993; Yao et al., 2004]. These

results suggest that germ cells in the ovary produce signals that are necessary for follicle survival and maturation, however, data from ovaries in which *Foxl2* is deleted after birth suggest otherwise. In these studies, germ cell deletion in mature follicles did not cause transdifferentiation of granulosa cells to Sertoli cells, suggesting that oocyte loss is more likely to be a consequence of somatic cell transdifferentiation than a cause [Uhlenhaut et al., 2009].

Oocytes are quiescent throughout the period of follicle formation, arrested in meiosis I. During fetal ovarian development, germ cells divide by mitosis from the time they invade the genital ridge until approximately 13.5 dpc [Monk and McLaren, 1981]. These early germ cells, termed oogonia, undergo a unique form of mitosis in which cytokinesis is incomplete, resulting in formation of a multi-nucleated germ cell nest [Pepling and Spradling, 1998]. Oogonia begin to enter meiosis around 13.5 dpc and are thereafter classified as oocytes. Meiotic initiation in the ovary is not synchronous; it begins in the anterior portion of the ovary and proceeds in a wave to the posterior pole [Menke et al., 2003; Yao et al., 2003; Bullejos and Koopman, 2004]. Oocytes advance through the leptotene, zygotene, and pachytene stages of meiosis I prophase I, then arrest in the diplotene stage between 17.5 dpc and the 5th day of postnatal life [Borum, 1961; McLaren, 2000]. During the first few days of postnatal life, pre-granulosa cells invade the germ cell nests and start to break down the nest structure. Pre-granulosa cells then enclose individual oocyte nuclei, resulting in formation of the primordial follicles [Merchant, 1975; Pepling and Spradling, 1998]. Finally, a thin layer of ex-

tracellular matrix is deposited around each follicle, creating an enclosed environment for oocyte maturation. Primordial follicles are located predominantly in the outer zone, termed the cortex, of the ovary.

Step 3: Compartmentalization of the Somatic Cell Environment

The somatic cell environment of the ovary must evolve and reorganize to support progression of a primordial follicle to the next stages of folliculogenesis, the primary, and later, the secondary and tertiary follicles. Changes in both the granulosa and theca cell lineages are essential to ovary compartmentalization. When a primordial follicle begins the transformation to become a primary follicle, pre-granulosa cells surrounding the follicle must also undergo a transformation that will enable them to perform their mature functions. Factors secreted by the oocyte stimulate pre-granulosa cells to change their morphology, from flattened and fibroblast-like to cuboidal, and switch from a quiescent to a proliferative state [Lintern-Moore and Moore, 1979]. As a result, granulosa cells begin to secrete factors involved in follicle development, such as stem cell factor (SCF/KIT) and AMH [Reynaud et al., 2000; Durlinger et al., 2002]. The oocyte within the primary follicle also secretes factors important for follicle maturation, such as the TGF β superfamily members growth differentiation factor 9 (GDF9) [Dong et al., 1996] and BMP15 [Yan et al., 2001; Su et al., 2004]. Over the course of follicle development, the granulosa cell population continues to grow and ultimately surrounds the oocyte with a layer at least 3 cells thick. The early stages of follicle development occur independently of pituitary gonadotropins; however, pituitary hormones are the primary regulators of granulosa cell proliferation and differentiation during later stages of folliculogenesis [for review see Edson et al., 2009].

Recruitment and activation of the theca cell lineage is thought to occur only in the mature follicle, following establishment of the granulosa cell lineage (see fig. 4). Theca cells are a mesenchymal cell type responsible for androgen production in the ovary and are essential for follicle growth. Compared to granulosa cells, little is known about theca cell specification. One reason for this is that currently no lineage markers exist that specifically recognize theca cells. Theca cells are believed to surround the developing follicle during the primary stage; however, they do not begin to express steroidogenic markers until the secondary or pre-antral stage

and therefore cannot be definitively identified until that time [Gelety and Magoffin, 1997]. Another unknown aspect of theca cell development is the origin of their precursor cells [reviewed by Young and McNeilly, 2010]. The most widely accepted theory of theca cell origins is that theca cells are recruited from the ovarian stroma by factors secreted by activated follicles [Erickson et al., 1985; Orisaka et al., 2006; Young and McNeilly, 2010]. As previously discussed, lineage tracing studies have established that no mesonephric cell migration into the ovary occurs between 11.5 and 16.5 dpc [Byskov and Lintern-Moore, 1973; Byskov, 1975, 1978; Martineau et al., 1997]; however, the possibility remains that cells from the mesonephros migrate into the ovary before or after this time and that these cells could contribute to the theca cell population. Preliminary findings from our group indicate that theca cells enter the ovary from the mesonephros during fetal life [Liu et al., in preparation]; however, the contribution of additional sources to the theca cell population cannot be excluded.

A combination of multiple factors, secreted by both the oocyte and granulosa cells of activated follicles, control the differentiation of theca cell precursors in the ovary. These include insulin-like growth factor (IGF1), basic fibroblast growth factor (bFGF), SCF/KIT, and the TGF β family members AMH and GDF9 [reviewed by Young and McNeilly, 2010]. Perhaps the most striking and specific theca cell defects are seen in mice lacking *Gdf9*. *Gdf9*-deficient follicles exhibit a complete failure to recruit theca cells and consequently, never progress past the type 3b primary follicle stage [Dong et al., 1996; Elvin et al., 1999]. Granulosa cells of *Gdf9*-deficient follicles also exhibit defects in KIT ligand secretion [Elvin et al., 1999]. KIT ligand is important for both theca cell differentiation and recruitment [Parrott and Skinner, 2000], and consequently it is not known if GDF9 is important to both of these processes, or just one.

Theca cells perform 2 critical functions in the developing follicle: they secrete androgens required for estrogen production and establish the vascular system that nourishes the growing follicle. Androgens produced by the theca cells are transported to the granulosa cells, which convert these androgens to estradiol [Rajah et al., 1992; reviewed by Young and McNeilly, 2010]. Small primordial follicles are located in the avascular region of the ovarian cortex and do not possess their own vascular system. Once the theca cell layer is established, the follicle develops a vascular network within the surrounding theca layer. This vascular network delivers essential nutrients to the follicle and circulates estradiol produced by neighbor-

ing granulosa cells to targets throughout the body. How this vasculature network is established and its role in folliculogenesis are currently under investigation by our lab.

Future Directions for Gonad Morphogenesis Research

The testes and ovaries perform analogous functions in males and females. Therefore, it is not surprising that these 2 organs are patterned by the common basic cellular events: lineage specification, migration, and vascularization. Despite the advancement made in understanding the process of testis and ovary morphogenesis, many questions remain to be answered. To start, the testes contain a unique cell type, the PTM cell, which does not appear to have a functional equivalent in the ovary. In the testis, PTM cells are essential for the deposition of the basal lamina around the testis cords. The basal lamina in the ovarian follicle is formed between the granulosa and theca layers without the need for a PTM-like cell type. It is possible that either the theca or granulosa cells perform the duties of the PTM cells in the ovary or that a unique cell type, for which specific markers do not exist, performs this role in the ovary.

Another glaring hole in the story of ovary development is found in the field of theca cell development. Neither the signals regulating theca cell differentiation nor the origins of theca cell precursors are presently known. Based on Leydig cell specification systems, the Hedgehog pathway is a potential regulator of theca cell specification [Yao et al., 2002; Barsoum et al., 2009]. *Dhh* is not expressed in the fetal ovary at any time and no ovarian phenotype has been reported for female mice lacking *Dhh* [Bitgood et al., 1996]; however, the possibility remains

that the hedgehog pathway may be involved in theca cell development in the adult ovaries. Evidence from bovine models suggests that theca cell precursors originate from the ovarian stroma [Orisaka et al., 2006]; however, significant species-specific differences are known to exist in ovarian development and it is possible that theca cells in mice and humans originate from a source other than the ovarian stroma. It is also possible that theca cells originate from more than 1 source, as seems to be the case for Sertoli cells in the testis.

Finally, the signals controlling enclosure of germ cells into testis cords or ovarian follicles remain woefully undefined. Germ cells and somatic cells act cooperatively to form the gamete-producing units of the gonad. How is it that germ cells 'know' to localize to the center of the cord/follicle, while somatic cells 'know' to form the outer layers of the cord/follicle? Presumably the XY germ cell expresses some sort of mark that allows it to distinguish its fellow germ cells from adjacent somatic cells, and the XX germ cell utilizes a similar system that also prevents multiple oocytes from joining the same follicle. How do potential pathways in the germ cells coordinate with additional signals from somatic cells? Sophisticated single-cell studies are required to answer many of these questions and as live-cell imaging technologies continue to improve, it is likely that answers to many of these questions will soon follow.

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References

- Adams IR, McLaren A: Sexually dimorphic development of mouse primordial germ cells: switching from oogenesis to spermatogenesis. *Development* 129:1155–1164 (2002).
- Albrecht KH, Eicher EM: Evidence that *Sry* is expressed in pre-Sertoli cells and Sertoli and granulosa cells have a common precursor. *Dev Biol* 240:92–107 (2001).
- Albrecht KH, Capel B, Washburn LL, Eicher EM: Defective mesonephric cell migration is associated with abnormal testis cord development in C57BL/6J XY (*Mus domesticus*) mice. *Dev Biol* 225:26–36 (2000).
- Archambeault DR, Yao HH: Activin A, a product of fetal Leydig cells, is a unique paracrine regulator of Sertoli cell proliferation and fetal testis cord expansion. *Proc Natl Acad Sci USA* 107:10526–10531 (2010).
- Bagheri-Fam S, Sim H, Bernard P, Jayakody I, Taketo MM, et al: Loss of *Fgfr2* leads to partial XY sex reversal. *Dev Biol* 314:71–83 (2008).
- Baron D, Cocquet J, Xia X, Fellous M, Guiguen Y, Veitia RA: An evolutionary and functional analysis of *FoxL2* in rainbow trout gonad differentiation. *J Mol Endocrinol* 33:705–715 (2004).
- Barsoum IB, Bingham NC, Parker KL, Jorgensen JS, Yao HH: Activation of the Hedgehog pathway in the mouse fetal ovary leads to ectopic appearance of fetal Leydig cells and female pseudohermaphroditism. *Dev Biol* 329:96–103 (2009).
- Behringer RR, Cate RL, Froelick GJ, Palmiter RD, Brinster RL: Abnormal sexual development in transgenic mice chronically expressing Mullerian inhibiting substance. *Nature* 345:167–170 (1990).
- Bitgood MJ, Shen L, McMahon AP: Sertoli cell signaling by Desert hedgehog regulates the male germline. *Curr Biol* 6:298–304 (1996).

- Bogani D, Siggers P, Brixey R, Warr N, Beddow S, et al: Loss of mitogen-activated protein kinase kinase 4 (MAP3K4) reveals a requirement for MAPK signalling in mouse sex determination. *PLoS Biol* 7(9): e1000196 (2009).
- Borum K: Oogenesis in the mouse. A study of the meiotic prophase. *Exp Cell Res* 24:495–507 (1961).
- Brennan J, Capel B: One tissue, two fates: molecular genetic events that underlie testis versus ovary development. *Nat Rev Genet* 5: 509–521 (2004).
- Brennan J, Tilmann C, Capel B: *Pdgfr*-alpha mediates testis cord organization and fetal Leydig cell development in the XY gonad. *Genes Dev* 17:800–810 (2003).
- Britt KL, Drummond AE, Dyson M, Wreford NG, Jones ME, et al: The ovarian phenotype of the aromatase knockout (ArKO) mouse. *J Steroid Biochem Mol Biol* 79:181–185 (2001).
- Buehr M, Gu S, McLaren A: Mesonephric contribution to testis differentiation in the fetal mouse. *Development* 117:273–281 (1993).
- Bullejos M, Koopman P: Spatially dynamic expression of *Sry* in mouse genital ridges. *Dev Dyn* 221:201–205 (2001).
- Bullejos M, Koopman P: Germ cells enter meiosis in a rostro-caudal wave during development of the mouse ovary. *Mol Reprod Dev* 68:422–428 (2004).
- Byskov AG: The role of the rete ovarii in meiosis and follicle formation in the cat, mink and ferret. *J Reprod Fertil* 45:201–209 (1975).
- Byskov AG: The anatomy and ultrastructure of the rete system in the fetal mouse ovary. *Biol Reprod* 19:720–735 (1978).
- Byskov AG, Lintern-Moore S: Follicle formation in the immature mouse ovary: the role of the rete ovarii. *J Anat* 116:207–217 (1973).
- Chassot AA, Ranc F, Gregoire EP, Roepers-Gajadien HL, Taketo MM, et al: Activation of beta-catenin signaling by *Rspo1* controls differentiation of the mammalian ovary. *Hum Mol Genet* 17:1264–1277 (2008).
- Clark AM, Garland KK, Russell LD: Desert hedgehog (*Dhh*) gene is required in the mouse testis for formation of adult-type Leydig cells and normal development of peritubular cells and seminiferous tubules. *Biol Reprod* 63:1825–1838 (2000).
- Colvin JS, Green RP, Schmahl J, Capel B, Ornitz DM: Male-to-female sex reversal in mice lacking fibroblast growth factor 9. *Cell* 104: 875–889 (2001).
- Combes AN, Wilhelm D, Davidson T, Dejana E, Harley V, et al: Endothelial cell migration directs testis cord formation. *Dev Biol* 326: 112–120 (2009).
- Cool J, Carmona FD, Szucsik JC, Capel B: Peritubular myoid cells are not the migrating population required for testis cord formation in the XY gonad. *Sex Dev* 2:128–133 (2008).
- Cool J, Defalco TJ, Capel B: Vascular-mesenchymal cross-talk through Vegf and Pdgf drives organ patterning. *Proc Natl Acad Sci USA* 108:167–172 (2011).
- Couse JF, Hewitt SC, Bunch DO, Sar M, Walker VR, et al: Postnatal sex reversal of the ovaries in mice lacking estrogen receptors alpha and beta. *Science* 286:2328–2331 (1999).
- Coveney D, Cool J, Oliver T, Capel B: Four-dimensional analysis of vascularization during primary development of an organ, the gonad. *Proc Natl Acad Sci USA* 105:7212–7217 (2008).
- Cupp AS, Kim GH, Skinner MK: Expression and action of neurotrophin-3 and nerve growth factor in embryonic and early postnatal rat testis development. *Biol Reprod* 63:1617–1628 (2000).
- Dong J, Albertini DF, Nishimori K, Kumar TR, Lu N, Matzuk MM: Growth differentiation factor-9 is required during early ovarian folliculogenesis. *Nature* 383:531–535 (1996).
- Durlinger AL, Gruijters MJ, Kramer P, Karels B, Ingraham HA, et al: Anti-Mullerian hormone inhibits initiation of primordial follicle growth in the mouse ovary. *Endocrinology* 143:1076–1084 (2002).
- Edson MA, Nagaraja AK, Matzuk MM: The mammalian ovary from genesis to revelation. *Endocr Rev* 30:624–712 (2009).
- Eicher EM, Shown EP, Washburn LL: Sex reversal in C57BL/6J-Y^{POS} mice corrected by a *Sry* transgene. *Philos Trans R Soc Lond B Biol Sci* 350:263–268; discussion 268–269 (1995).
- Elvin JA, Yan C, Wang P, Nishimori K, Matzuk MM: Molecular characterization of the follicle defects in the growth differentiation factor 9-deficient ovary. *Mol Endocrinol* 13: 1018–1034 (1999).
- Erickson GF, Magoffin DA, Dyer CA, Hofeditz C: The ovarian androgen producing cells: a review of structure/function relationships. *Endocr Rev* 6:371–399 (1985).
- Gelety TJ, Magoffin DA: Ontogeny of steroidogenic enzyme gene expression in ovarian theca-interstitial cells in the rat: regulation by a paracrine theca-differentiating factor prior to achieving luteinizing hormone responsiveness. *Biol Reprod* 56:938–945 (1997).
- Ginsburg M, Snow MH, McLaren A: Primordial germ cells in the mouse embryo during gastrulation. *Development* 110:521–528 (1990).
- Gubbay J, Collignon J, Koopman P, Capel B, Economou A, et al: A gene mapping to the sex-determining region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes. *Nature* 346: 245–250 (1990).
- Hacker A, Capel B, Goodfellow P, Lovell-Badge R: Expression of *Sry*, the mouse sex determining gene. *Development* 121:1603–1614 (1995).
- Hadley MA, Byers SW, Suarez-Quian CA, Kleinman HK, Dym M: Extracellular matrix regulates Sertoli cell differentiation, testicular cord formation, and germ cell development in vitro. *J Cell Biol* 101:1511–1522 (1985).
- Hashimoto N, Kubokawa R, Yamazaki K, Noguchi M, Kato Y: Germ cell deficiency causes testis cord differentiation in reconstituted mouse fetal ovaries. *J Exp Zool* 253:61–70 (1990).
- Hawkins JR, Taylor A, Berta P, Levilliers J, van der Auwera B, Goodfellow PN: Mutational analysis of *SRY*: nonsense and missense mutations in XY sex reversal. *Hum Genet* 88: 471–474 (1992).
- Hirshfield AN: Development of follicles in the mammalian ovary. *Int Rev Cytol* 124:43–101 (1991).
- Karl J, Capel B: Sertoli cells of the mouse testis originate from the coelomic epithelium. *Dev Biol* 203:323–333 (1998).
- Kent J, Wheatley SC, Andrews JE, Sinclair AH, Koopman P: A male-specific role for *SOX9* in vertebrate sex determination. *Development* 122:2813–2822 (1996).
- Kim Y, Kobayashi A, Sekido R, Dinapoli L, Brennan J, et al: *Fgf9* and *Wnt4* act as antagonistic signals to regulate mammalian sex determination. *PLoS Biol* 4:e187 (2006).
- Koopman P, Munsterberg A, Capel B, Vivian N, Lovell-Badge R: Expression of a candidate sex-determining gene during mouse testis differentiation. *Nature* 348:450–452 (1990).
- Koopman P, Gubbay J, Vivian N, Goodfellow P, Lovell-Badge R: Male development of chromosomally female mice transgenic for *Sry*. *Nature* 351:117–121 (1991).
- Lintern-Moore S, Moore GP: The initiation of follicle and oocyte growth in the mouse ovary. *Biol Reprod* 20:773–778 (1979).
- Liu CF, Liu C, Yao HH: Building pathways for ovary organogenesis in the mouse embryo. *Curr Top Dev Biol* 90:263–290 (2010a).
- Liu CF, Parker K, Yao HH: *WNT4*/beta-catenin pathway maintains female germ cell survival by inhibiting activin betaB in the mouse fetal ovary. *PLoS One* 5:e10382 (2010b).
- Lovell-Badge R, Robertson E: XY female mice resulting from a heritable mutation in the primary testis-determining gene, *Tdy*. *Development* 109:635–646 (1990).
- Manuylov NL, Smagulova FO, Leach L, Tevosian SG: Ovarian development in mice requires the GATA4-FOG2 transcription complex. *Development* 135:3731–3743 (2008).
- Martineau J, Nordqvist K, Tilmann C, Lovell-Badge R, Capel B: Male-specific cell migration into the developing gonad. *Curr Biol* 7: 958–968 (1997).
- McLaren A: Meiosis and differentiation of mouse germ cells. *Symp Soc Exp Biol* 38:7–23 (1984).
- McLaren A: Development of the mammalian gonad: the fate of the supporting cell lineage. *Bioessays* 13:151–156 (1991).
- McLaren A: Germ and somatic cell lineages in the developing gonad. *Mol Cell Endocrinol* 163:3–9 (2000).
- Menke DB, Koubova J, Page DC: Sexual differentiation of germ cells in XX mouse gonads occurs in an anterior-to-posterior wave. *Dev Biol* 262:303–312 (2003).

- Merchant H: Rat gonadal and ovarian organogenesis with and without germ cells. An ultrastructural study. *Dev Biol* 44:1–21 (1975).
- Merchant-Larios H, Centeno B: Morphogenesis of the ovary from the sterile W/Wv mouse. *Prog Clin Biol Res* 59B:383–392 (1981).
- Moniot B, Declosmenil F, Barrionuevo F, Scherer G, Aritake K, et al: The PGD2 pathway, independently of FGF9, amplifies SOX9 activity in Sertoli cells during male sexual differentiation. *Development* 136:1813–1821 (2009).
- Monk M, McLaren A: X-chromosome activity in foetal germ cells of the mouse. *J Embryol Exp Morphol* 63:75–84 (1981).
- Mork L, Maatouk DM, McMahon JA, Guo JJ, Zhang P, et al: Temporal differences in granulosa cell specification in the ovary reflect distinct follicle fates in mice. *Biol Reprod* 86:37 (2012).
- Nel-Themaat L, Vadakkan TJ, Wang Y, Dickinson ME, Akiyama H, Behringer RR: Morphometric analysis of testis cord formation in *Sox9-EGFP* mice. *Dev Dyn* 238:1100–1110 (2009).
- Orisaka M, Tajima K, Mizutani T, Miyamoto K, Tsang BK, et al: Granulosa cells promote differentiation of cortical stromal cells into theca cells in the bovine ovary. *Biol Reprod* 75:734–740 (2006).
- Ottolenghi C, Pelosi E, Tran J, Colombino M, Douglass E, et al: Loss of *Wnt4* and *Foxl2* leads to female-to-male sex reversal extending to germ cells. *Hum Mol Genet* 16:2795–2804 (2007).
- Parma P, Radi O, Vidal V, Chaboissier MC, Delambra E, et al: R-spondin1 is essential in sex determination, skin differentiation and malignancy. *Nat Genet* 38:1304–1309 (2006).
- Parrott JA, Skinner MK: Kit ligand actions on ovarian stromal cells: effects on theca cell recruitment and steroid production. *Mol Reprod Dev* 55:55–64 (2000).
- Pepling ME, Spradling AC: Female mouse germ cells form synchronously dividing cysts. *Development* 125:3323–3328 (1998).
- Pierucci-Alves F, Clark AM, Russell LD: A developmental study of the Desert hedgehog-null mouse testis. *Biol Reprod* 65:1392–1402 (2001).
- Pisarska MD, Barlow G, Kuo FT: Minireview: Roles of the forkhead transcription factor FOXL2 in granulosa cell biology and pathology. *Endocrinology* 152:1199–1208 (2011).
- Pollack SE, Furth EE, Kallen CB, Arakane F, Kiriakidou M, et al: Localization of the steroidogenic acute regulatory protein in human tissues. *J Clin Endocrinol Metab* 82:4243–4251 (1997).
- Rajah R, Glaser EM, Hirshfield AN: The changing architecture of the neonatal rat ovary during histogenesis. *Dev Dyn* 194:177–192 (1992).
- Reynaud K, Cortvrindt R, Smits J, Driancourt MA: Effects of Kit ligand and anti-Kit antibody on growth of cultured mouse preantral follicles. *Mol Reprod Dev* 56:483–494 (2000).
- Ross AJ, Tilman C, Yao H, MacLaughlin D, Capel B: AMH induces mesonephric cell migration in XX gonads. *Mol Cell Endocrinol* 211:1–7 (2003).
- Sawyer HR, Smith P, Heath DA, Juengel JL, Wakefield SJ, McNatty KP: Formation of ovarian follicles during fetal development in sheep. *Biol Reprod* 66:1134–1150 (2002).
- Schmahl J, Capel B: Cell proliferation is necessary for the determination of male fate in the gonad. *Dev Biol* 258:264–276 (2003).
- Schmahl J, Eicher EM, Washburn LL, Capel B: *Sry* induces cell proliferation in the mouse gonad. *Development* 127:65–73 (2000).
- Schmahl J, Kim Y, Colvin JS, Ornitz DM, Capel B: *Fgf9* induces proliferation and nuclear localization of FGFR2 in Sertoli precursors during male sex determination. *Development* 131:3627–3636 (2004).
- Schmidt D, Ovitt CE, Anlag K, Fehsenfeld S, Gredsted L, et al: The murine winged-helix transcription factor *Foxl2* is required for granulosa cell differentiation and ovary maintenance. *Development* 131:933–942 (2004).
- Sekido R, Lovell-Badge R: Sex determination involves synergistic action of SRY and SF1 on a specific *Sox9* enhancer. *Nature* 453:930–934 (2008).
- Sinclair AH, Berta P, Palmer MS, Hawkins JR, Griffiths BL, et al: A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. *Nature* 346:240–244 (1990).
- Skinner MK, Tung PS, Fritz IB: Cooperativity between Sertoli cells and testicular peritubular cells in the production and deposition of extracellular matrix components. *J Cell Biol* 100:1941–1947 (1985).
- Su YQ, Wu X, O'Brien MJ, Pendola FL, Denegre JN, et al: Synergistic roles of BMP15 and GDF9 in the development and function of the oocyte-cumulus cell complex in mice: genetic evidence for an oocyte-granulosa cell regulatory loop. *Dev Biol* 276:64–73 (2004).
- Taketo T, Saeed J, Manganaro T, Takahashi M, Donahoe PK: Mullerian inhibiting substance production associated with loss of oocytes and testicular differentiation in the transplanted mouse XX gonadal primordium. *Biol Reprod* 49:13–23 (1993).
- Tilmann C, Capel B: Mesonephric cell migration induces testis cord formation and Sertoli cell differentiation in the mammalian gonad. *Development* 126:2883–2890 (1999).
- Tomizuka K, Horikoshi K, Kitada R, Sugawara Y, Iba Y, et al: R-spondin1 plays an essential role in ovarian development through positively regulating Wnt-4 signaling. *Hum Mol Genet* 17:1278–1291 (2008).
- Tung PS, Fritz IB: Extracellular matrix components and testicular peritubular cells influence the rate and pattern of Sertoli cell migration in vitro. *Dev Biol* 113:119–134 (1986).
- Uda M, Ottolenghi C, Crisponi L, Garcia JE, Deiana M, et al: *Foxl2* disruption causes mouse ovarian failure by pervasive blockage of follicle development. *Hum Mol Genet* 13:1171–1181 (2004).
- Uhlenhaut NH, Jakob S, Anlag K, Eisenberger T, Sekido R, et al: Somatic sex reprogramming of adult ovaries to testes by FOXL2 ablation. *Cell* 139:1130–1142 (2009).
- Vainio S, Heikkila M, Kispert A, Chin N, McMahon AP: Female development in mammals is regulated by Wnt-4 signalling. *Nature* 397:405–409 (1999).
- Wainwright EN, Wilhelm D: The game plan: cellular and molecular mechanisms of mammalian testis development. *Curr Top Dev Biol* 90:231–262 (2010).
- Wartenberg H: Human testicular development and the role of the mesonephros in the origin of a dual Sertoli cell system. *Andrologia* 10:1–21 (1978).
- Wilhelm D, Martinson F, Bradford S, Wilson MJ, Combes AN, et al: Sertoli cell differentiation is induced both cell-autonomously and through prostaglandin signaling during mammalian sex determination. *Dev Biol* 287:111–124 (2005).
- Yan C, Wang P, Demayo J, Demayo FJ, Elvin JA, et al: Synergistic roles of bone morphogenetic protein 15 and growth differentiation factor 9 in ovarian function. *Mol Endocrinol* 15:854–866 (2001).
- Yao HH, Capel B: Disruption of testis cords by cyclopamine or forskolin reveals independent cellular pathways in testis organogenesis. *Dev Biol* 246:356–365 (2002).
- Yao HH, Whoriskey W, Capel B: Desert Hedgehog/Patched 1 signaling specifies fetal Leydig cell fate in testis organogenesis. *Genes Dev* 16:1433–1440 (2002).
- Yao HH, Dinapoli L, Capel B: Meiotic germ cells antagonize mesonephric cell migration and testis cord formation in mouse gonads. *Development* 130:5895–5902 (2003).
- Yao HH, Matzuk MM, Jorgez CJ, Menke DB, Page DC, et al: Follistatin operates downstream of Wnt4 in mammalian ovary organogenesis. *Dev Dyn* 230:210–215 (2004).
- Yao HH, Aardema J, Holthuisen K: Sexually dimorphic regulation of inhibin beta B in establishing gonadal vasculature in mice. *Biol Reprod* 74:978–983 (2006).
- Young JM, McNeilly AS: Theca: the forgotten cell of the ovarian follicle. *Reproduction* 140:489–504 (2010).