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Building Pathways for Ovary Organogenesis in the Mouse Embryo

Chia-Feng Liu*, Chang Liu†, and Humphrey H-C Yao*

*Department of Veterinary Biosciences, University of Illinois at Urbana-Champaign, Illinois, USA

†Department of Animal Science, University of Illinois at Urbana-Champaign, Illinois, USA

Abstract

Despite its significant role in oocyte generation and hormone production in adulthood, the ovary, with regard to its formation, has received little attention compared to its male counterpart, the testis. With the exception of germ cells, which undergo a female-specific pattern of meiosis, morphological changes in the fetal ovary are subtle. Over the past 40 years, a number of hypotheses have been proposed for the organogenesis of the mammalian ovary. It was not until the turn of the millennium, thanks to the advancement of genetic and genomic approaches, that pathways for ovary organogenesis that consist of positive and negative regulators have started to emerge. Through the action of secreted factors (R-spondin1, WNT4, and follistatin) and transcription regulators (β -catenin and FOXL2), the developmental fate of the somatic cells is directed toward ovarian, while testicular components are suppressed. In this chapter, we review the history of studying ovary organogenesis in mammals and present the most recent discoveries using the mouse as the model organism.

1. Evolution of the Hypotheses for Ovary Organogenesis in Mammals

The importance of the ovary in mammalian reproduction was not recognized until von Baer's discovery that ovarian follicles are the source of mammalian eggs (von Baer, 1827). Known as "the testicle of the female" before the 16th century, the ovary was considered merely a structure insignificant to generation of species (Harvey, 1653) or a gland that produced female "semen" (Descartes, 1664; Le Grand, 1672; Wharton, 1656). Revelation of the egg-producing ability and endocrine capability of the ovary transformed scientists' view on its role in reproduction. Since then, the ovary has become the centerpiece of the female reproductive system.

The structural and functional foundation of the ovary is established during embryonic development in most eutherian or placental mammals. At the time of fertilization, the sex of the embryo is determined when the sperm carrying either an X or Y chromosome fertilizes the oocyte, which contains one X chromosome. The Y chromosome and its testis-determining element play an indisputable role in testis formation (see Chapter 2 on testis development). However, the number of X chromosomes is irrelevant to the establishment of the ovary, as humans and mice with XO aneuploidy still develop ovaries (Morris, 1968; Ohno and Cattanaach, 1962; Singh and Carr, 1966, 1967; Welshons and Russell, 1959).

Alfred Jost, in his groundbreaking work in the early 1950s, revealed the relationship between the sexes of the gonads and phenotypic sexual characteristics. Jost discovered that when the gonads of either sex were removed before the onset of sexual differentiation, female internal and external sexual characteristics arose regardless of the chromosomal sex of the embryo. As a result it was concluded that the female sexual phenotypes appear by default, independent of the presence of gonads (Jost *et al.*, 1953). Jost later extended this paradigm to gonadal differentiation and proposed that a putative "male organizer" prevents

the gonadal primordium from developing into an ovary and forces it to become a testis (Jost, 1972) (Fig. 7.1).

The mechanism of ovary organogenesis was explored further by Eicher and Washburn in 1983. Based on the observation of a strain of XY mice where testes were sex-reversed to ovaries (Eicher and Washburn, 1983; Washburn and Eicher, 1983), they proposed that an ovary-determining gene (or *Od*), located on an autosome or the X chromosome, initiates ovary differentiation. In the male embryo, the *Od* gene and subsequent ovary differentiation are inhibited by the testis-determining gene on the Y chromosome (or *Tdy*), which presumably gains its functions preceding the *Od* gene (Fig. 7.1). The discovery of the *SRY* gene (Sex-determining region on the Y chromosome) in the early 1990s confirmed the identity of *Tdy* and revealed its dominant role in testis determination. However, the puzzling cases of XX male in humans and the polled intersex syndrome (PIS) XX goats, where testes develop in females without *SRY* or any Y chromosome fragments, kindled a rethinking of the mechanism for ovary differentiation. To explain these cases, McElreavey and colleagues proposed the Z hypothesis that in normal XX gonads the Z gene suppresses the emergence of testis program (Fig. 7.1). Loss-of-function of the Z gene in the XX gonad, therefore, results in formation of the testis (McElreavey *et al.*, 1993). In contrast in normal XY gonads, the *SRY* gene antagonizes the functions of the Z gene, allowing the progression of the testis program (Fig. 7.1).

At the turn of the 21st century, mouse genetic models and human clinical cases have made the case that the mechanism for ovary differentiation is beyond X and Y chromosomes and the Z factor. Complicated antagonism and synergism at the levels of cell–cell interaction and transcriptional regulation have already occurred in the undifferentiated ovary. New findings also provide insights into sexually dimorphic regulation of germ cell meiosis and prompt a paradigm shift in our views on how the somatic environment and female germ cells interact. In this chapter, we review the current knowledge of ovary organogenesis using mouse models as the core and make comparisons to humans.

2. Building the Foundation: Morphogenesis of the Ovary

The genital ridge (or gonadal primordium), the structural precursor of both the testis and the ovary, emerges as a thickening of the coelomic epithelium that overlays the ventral aspect of the mesonephros at 10 days *post coitum* (dpc) in the mouse embryo. In both sexes, primordial germ cells (PGCs), which originate from the proximal epiblast, migrate through the wall of the hindgut at ~9 dpc and into the genital ridge at 10.5–11.5 dpc (Anderson *et al.*, 2000; Molyneaux *et al.*, 2001; Tam and Snow, 1981). Once the PGCs colonize the genital ridges, clusters of PGCs coalesce with somatic cells and form the ovigerous cords, which are delineated by the deposition of the basal lamina (Merchant, 1975; Pepling and Spradling, 1998; Ruby *et al.*, 1969). In the developing testis, ovigerous cords differentiate into well-defined tubule structures known as testis cords as a result of the action of Sertoli cells (see Chapter 2 on testis development). In contrast in the fetal ovary, ovigerous cords remain as clusters of female germ cells (or germ cell nests) that are surrounded loosely by somatic cells (Fig. 7.2). Around the time of birth, somatic cells start to break down the germ cell nests by enclosing individual female germ cells or oocytes, leading to the formation of primordial follicles (Merchant-Larios and Chimal-Monroy, 1989; Pepling and Spradling, 1998). Germ cell nests and the forming primordial follicles populate predominantly the outer zone or cortex of the fetal ovary.

In the adult ovary, development of follicles occurs mainly in the ovarian cortex, whereas the medulla is the structure where the vasculature and nerves enter. Ovarian follicles in the cortex consist of oocytes and surrounding somatic cells including granulosa and theca cells.

Granulosa cells, an epithelial cell type that forms connections with the oocytes during the fetal stage, support oocyte development and produce hormones responsible for the development and maintenance of the female reproductive system. Theca cells, a mesenchymal cell type that appear only postnatally, are the major source of androgens, which are ultimately converted to estrogens by the granulosa cells (Erickson *et al.*, 1985; Quattropani, 1973).

Granulosa cells in the ovary and Sertoli cells in the testis are derived from common somatic precursor cells in the genital ridge, at least in mice (Albrecht and Eicher, 2001; McLaren, 1991, 2000). Based on morphological and histological observations, granulosa cell precursors could originate from three possible sources (Fig. 7.2): rete ovarii connecting to the neighboring mesonephros (Byskov, 1975, 1978; Byskov and Lintern-Moore, 1973; Byskov and Rasmussen, 1973; Zamboni *et al.*, 1975), the existing mesenchymal cells in the genital ridge (Albrecht and Eicher, 2001; Pinkerton *et al.*, 1961), or ovarian surface epithelium (Gondos, 1975; Motta and Makabe, 1982; Sawyer *et al.*, 2002). Species variation seems evident in regard to the cellular origin(s) of granulosa cell precursors and no definitive sources have been identified in species other than mice. The possible contribution of multiple origins to the granulosa cell lineage cannot be excluded.

Theca cells, the ovarian counterpart of Leydig cells in the testis, begin to appear in the postnatal ovary. Theca cells are thought to be derived from fibroblast-like precursors in the ovarian stroma (Erickson *et al.*, 1985; Hirshfield, 1991; Quattropani, 1973). As the ovarian follicles develop to the secondary stage with multiple layers of granulosa cells surrounding the oocyte, stromal cells adjacent to the basal lamina form a layer of elongated cells. This layer is known as the theca interna, which is a highly vascularized steroidogenic tissue. Outside of the theca interna, a loosely organized band of nonsteroidogenic cells or theca externa is formed. Theca cells are only found in the developing follicle and are adjacent to granulosa cells; therefore, their differentiation is considered to be under the control of granulosa cells (Kotsuji *et al.*, 1990; Orisaka *et al.*, 2006). This concept is supported by the findings that small-molecular-weight proteins enriched from secretions of developing follicles stimulate theca cell differentiation (Magoffin and Magarelli, 1995).

3. Making Eggs: Establishment of the Female Germline

Generation of the female germline (or oogenesis) is the one of the other key functions of the ovary in addition to hormone production. Between 1920 and 1950, the field of oogenesis was dominated by the doctrine that the germinal epithelium, or ovarian surface epithelium encapsulating the ovary, gave rise to oocytes during each estrous or menstrual cycle. This doctrine was later rejected based on evidence that a finite stock of meiotic oocytes is present in the ovary before birth and no new oocytes are generated during adult life (Zuckerman, 1951). However, the discovery of putative germline stem cells in postnatal ovaries led to the resurgence of the controversial idea of “neo-oogenesis” (Johnson *et al.*, 2005, 2004; Zou *et al.*, 2009). In this chapter, we focus only on the establishment of female germline during fetal life.

In the mouse fetal ovary, oocytes start to form around 13.5 dpc when female germ cells (or oogonia) stop proliferating and enter the first meiosis (McLaren, 2000). The oocytes progress through leptotema, zygotema, pachynema, and diplonema and eventually rest at the dictyate stage of meiotic prophase I around the time of birth (Borum, 1961; Speed, 1982). Oocytes do not resume meiosis until ovulation when the female reaches sexual maturity. Once ovulated from the ovary, oocytes complete the first meiotic division, enter the second meiotic division, and arrest again. The second meiotic division is completed after fertilization (Lewis *et al.*, 2006).

Germ cells in the mouse testis behave very differently from their female counterparts. Instead of entering meiosis at 13.5 dpc, male germ cells arrest in mitosis in fetal life and resume mitosis immediately after birth (Hilscher *et al.*, 1974). The male germ cells or spermatogonia then undergo the first meiotic and second meiotic divisions to generate spermatids. The process is repeated many times to constitute a renewing supply of mature sperm (Lewis *et al.*, 2006). In contrast to the finite stock of oocytes at the time of birth, spermatogonia in the testis retain the ability to self-renew throughout the entire reproductive life.

How germ cells make the decision to follow the female or male path has been a central focus of study since 1970s. By creating an XX/XY chimeric embryo, researchers observed that XY germ cells in the ovarian tissue enter meiosis and become functional Y-bearing oocytes (Evans *et al.*, 1977; McLaren *et al.*, 1972; Mystkowska and Tarkowski, 1970). By contrast, XX germ cells avoid entering meiosis if they find themselves in a testicular environment (Palmer and Burgoyne, 1991). The conclusion was, therefore, drawn that all germ cells, regardless of their sex chromosome constitution, are programmed to follow male or female pattern of meiosis according to the surrounding somatic environment (McLaren, 1995, 2003).

Byskov and others proposed that germ cell meiosis is controlled by meiosis-inducing substance or/and a meiosis-preventing substance produced by the somatic cells in the gonads (Byskov, 1974; Byskov *et al.*, 1995, 1998; Byskov *et al.*, 1993; Byskov and Saxen, 1976; Gondos *et al.*, 1996; Grinstead and Byskov, 1981). When an undifferentiated fetal testis is cultured with ovaries containing meiotic germ cells, the male germ cells in the testis are coaxed into entering meiosis (Byskov and Saxen, 1976). On the other hand, when ovaries containing germ cells in meiosis are cultured with fetal testes with well-formed testicular structure, the oocytes are prevented from reaching the diplotene stage of meiotic prophase. Thus it was hypothesized that the fetal ovary secretes a “meiosis-inducing substance”, which triggers the meiotic entry of germ cells. The fetal testis instead produces a “meiosis-inhibiting substance” that prevents germ cells from entering meiosis (Byskov and Saxen, 1976).

In contrast to the hypothesis that the ovarian somatic environment secretes factor(s) that induces germ cell meiosis, McLaren and others were in favor of the concept that germ cell entry into meiosis follows a cell-autonomous or intrinsic program (McLaren, 1984; McLaren and Southee, 1997). This concept was based on the observation that when male germ cells lose their way during migration and settle in nongonadal organs such as mesonephros and adrenal, these stray germ cells enter meiosis following the same developmental time frame as their female counterparts in the ovary (Chuma and Nakatsuji, 2001; McLaren, 1983; McLaren and Southee, 1997; Upadhyay and Zamboni, 1982; Zamboni and Upadhyay, 1983). Likewise, XY germ cells enter and progress through meiotic prophase after they are separated from Sertoli cells and then cultured with other nongonadal somatic cells such as embryonic lung cells (McLaren and Southee, 1997). These findings led to the hypothesis that germ cells in the fetal ovary enter meiosis spontaneously, whereas meiosis is inhibited in the testis by factors produced by the somatic cells, probably Sertoli cells (Donovan *et al.*, 1986; McLaren and Southee, 1997).

The debate on how sexually dimorphic pattern of germ cell meiosis is established was resolved by the findings of Bowles *et al.* and Koubova *et al.* in 2006. A mechanism involving retinoic acid (RA) and its degrading enzyme CYP26B1 is in action, with both meiosis-inducing (RA) and meiosis-inhibiting (CYP26B1) properties (Fig. 7.3). The first clue that RA might play a role in meiotic entry of germ cells in the ovary came from an expression screen designed to identify sexually dimorphic genes in mouse fetal gonads. It

was found that *Cyp26b1*, the gene encoding a P450 enzyme that degrades RA (Hernandez *et al.*, 2007; Romand *et al.*, 2006; White *et al.*, 2000; Yashiro *et al.*, 2004), shows a testis-specific expression pattern. Initially present in gonads of both sexes, *Cyp26b1* becomes undetectable in female gonads after 11.5 dpc. However in the testis, *Cyp26b1* expression is maintained and reaches its maximum at 13.5 dpc (Bowles *et al.*, 2006). The presence of RA-degrading CYP26b1 in fetal testes is consistent with a significant lower level of RA in the testis compared to the ovary at 13.5 dpc. This evidence suggests that a low RA level is necessary for preventing germ cell meiosis in the testis (Bowles *et al.*, 2006). In other words, a high RA level in the fetal ovary is responsible for inducing germ cell entry into meiosis. Indeed, when exogenous RA is given to fetal testes in culture, XY germ cells enter meiosis (Bowles *et al.*, 2006; Koubova *et al.*, 2006). In addition, treatment of fetal testes with CYP26 inhibitors in culture induces meiotic entry of XY germ cells and an upregulation of stimulated by retinoic acid gene 8 (*Stra8*), which is required for premeiotic DNA replication and the subsequent events of meiotic prophase in germ cells of embryonic ovaries (Baltus *et al.*, 2006; Bowles *et al.*, 2006). Finally, exposure of fetal ovaries to RA antagonists prevents XX germ cells from entering meiosis (Bowles *et al.*, 2006). These *in vitro* results were later substantiated by examination of the *Cyp26b1* knockout mice. In the absence of functional *Cyp26b1* genes, RA levels are increased in embryonic testes and XY germ cells enter meiosis prophase at 13.5 dpc, similar to germ cells in a normal fetal ovary (Bowles *et al.*, 2006; MacLean *et al.*, 2007). Collectively, this evidence supports the concept that a high RA in the fetal ovary due to lack of CYP26B1 is responsible for inducing germ cell meiosis (Fig. 7.3) (Bowles and Koopman, 2007). Presence of CYP26B1 in the fetal testis prevents accumulation of RA and consequent germ cell meiosis.

Intriguingly, fetal testes and ovaries are not the source of RA. Gonads apparently lack the ability to synthesize RA because they do not express *Aldh1a2*, the gene encoding the major enzyme for RA synthesis (Bowles *et al.*, 2006). Instead, RA is secreted by mesonephroi, the mesoderm-derived tissues immediately adjacent to the gonads (Bowles *et al.*, 2006). In 1970s, Byskov proposed that rete ovarii, the extending mesonephric derivative that connects to the ovarian medulla, may be the source of “meiosis-inducing factor” (Byskov, 1974, 1975; Byskov and Lintern-Moore, 1973). In the mouse gonads, mesonephric tubules connect to the anterior portion of the gonads. If indeed the meiosis-inducing factor (or RA) comes from the mesonephric tubules, one would expect that the RA level would be higher at the anterior end than at the posterior end of the gonad. This anterior-to-posterior gradient of RA in the gonads was later confirmed (Fig. 7.3) (Bowles *et al.*, 2006). This phenomenon is also supported by the fact that female germ cells in the anterior part of the fetal ovary enter meiosis earlier than those in the posterior end of the fetal ovary (Bullejos and Koopman, 2004; Menke and Page, 2002; Yao *et al.*, 2003).

Germ cell meiosis in the fetal ovary is controlled by not only the availability of the meiosis-inducing RA, but also the competence of germ cells to respond to RA. Germline specific RNA-binding protein DAZL (deleted in azoospermia-like gene) and NANOS2 have emerged as intrinsic factors in germ cells that define their ability to enter meiosis in response to RA. When *Dazl* became nonfunctional, germ cells in the fetal ovary fail to enter meiosis. In addition, male germ cells in the *Dazl* knockout testis lose their ability to enter meiosis in response to exogenous RA (Lin *et al.*, 2008). This evidence implies that the presence of *Dazl* is a prerequisite for germ cells to gain the ability to respond to RA, therefore, becoming meiosis-competent. *Nanos2*, on the other hand, plays a role in suppressing germ cell meiosis. *Nanos2* is expressed exclusively in germ cells in the fetal testis, whereas it is absent in female germ cells (Tsuda *et al.*, 2003). Loss of *Nanos2* in the fetal testis results in upregulation of *Stra8* and meiosis of male germ cells. Ectopic expression of *Nanos2* in the female germ cells decreases *Stra8* expression and inhibits germ cell meiosis (Suzuki and

Saga, 2008). NANOS2 probably inhibits germ cell meiosis by decreasing *Stra8* expression, the downstream target of RA (Suzuki *et al.*, 2010).

In summary, establishment of the female germline, characterized by entry into meiosis in fetal life, requires a synchronized action of both extracellular and intracellular factors. Extrinsic RA, derived from the mesonephros, serves as a meiosis-inducing agent in the fetal ovary. Female germ cells become competent to enter meiosis in response to RA only after they are primed by the presence of intrinsic factor DAZL. The fetal ovary also suppresses production of the meiosis-inhibiting factors including *Cyp26b1* and *Nanos2*, clearing the path for female germ cells to differentiate into oocytes. The observation of putative female germline stem cells in adult ovaries (Johnson *et al.*, 2004, 2005; Zou *et al.*, 2009) raises the question of how these putative germline stem cells escape from the meiosis-inducing RA during fetal development and re-enter the meiosis path later in life. Knowledge of sexually dimorphic regulation of the germline could have implications for solving the controversy around the existence of female germline stem cells in adult ovaries.

4. Determination of the Ovarian Identity: Differentiation of Granulosa Cells

Lessons from regulation of germ cell meiosis highlight the importance of the somatic cell environment in ovary differentiation. In contrast to female germ cells, somatic cells in the fetal ovary have received little attention because their differentiation lacks dramatic elements compared to their counterparts in the fetal testis. The *Sry*-expressing Sertoli cells in the testis orchestrate morphological transformation of the testis (see Chapter 2 on testis development). Granulosa cells, the main somatic cells in the developing ovary, are derived from the same progenitor cells as the Sertoli cells. It was originally thought that the progenitor cells differentiate into granulosa cells by default when the *Sry* is absent, as in XX animals (Fig. 7.1). Influenced by the hypotheses of ovary-determining genes and the Z factor (Fig. 7.1), researchers began to search for genes exclusively expressed in the somatic cells in the ovary. Functional genetic analyses have identified many ovary-specific and somatic cell-derived genes that can be classified into two categories: intracellular factors such as transcription factors (DAX1, FOXL2, and β -catenin) and extracellular factors with paracrine and/or autocrine properties (R-spondin1 and WNT4).

Dax1 (dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1) and *Foxl2* were once the prime candidates for ovary organogenesis and establishment of granulosa cell lineage. *DAX1* was initially considered as the ovary-determining gene because of its X-linked nature and function as a transcriptional regulator (Swain *et al.*, 1998). However, null mutation of *Dax1* in female mouse embryos did not affect formation and development of the ovary (Meeks *et al.*, 2003a-c; Yu *et al.*, 1998). Instead of being an ovary-determining gene, *Dax1* is found to have the anti-testis or Z property in the fetal testis. Duplication of a small piece of the X chromosome that contains *DAX1* in XY humans leads to testis-to-ovary sex reversal (Zanaria *et al.*, 1994). Transgenic mice carrying multiple copies of *Dax1* genes also have testis-to-ovary sex reversal (Swain *et al.*, 1998), suggesting a dose-dependent, anti-testis role of DAX1.

FOXL2, a member of the forkhead transcription factor family, gained attention because of its potential link to the ovary-to-testis sex reversal in the PIS XX goats (Pailhoux *et al.*, 2001, 2002). *FOXL2*, an autosomal gene, shows a granulosa cell-specific pattern conserved among vertebrate species (Loffler *et al.*, 2003; Wang *et al.*, 2004). Originally thought to be the candidate Z factor, *FOXL2* was later found not to be involved in early ovary organogenesis at least in humans and mice. In the absence of functional *FOXL2* genes, human and mouse females develop granulosa cell defects and signs of premature ovarian failure postnatally; however, no signs of ovary-to-testis sex reversal are observed as

predicted by the Z hypothesis (Ottolenghi *et al.*, 2005; Schmidt *et al.*, 2004). *FOXL2* and probably *DAX1* may not be critical for early ovary formation, but the possibility of synergy or compensation by other factors cannot be excluded, as discussed below.

In contrast to the uncertain roles of *DAX1* and *FOXL2*, secreted factor R-spondin1 (*RSPO1*) and *WNT4* are doubtlessly critical for establishment of the somatic cell environment in the fetal ovary. Initially expressed in somatic cells in gonads of both sexes, *Rspo1* and *Wnt4* expression become ovary-specific after the time of sex determination (Chassot *et al.*, 2008b; Parma *et al.*, 2006; Tomizuka *et al.*, 2008; Vainio *et al.*, 1999). Female mouse embryos lacking functional *Rspo1* or *Wnt4* develop similar ovarian defects including formation of ectopic testis vasculature, appearance of androgen-producing cells, loss of female germ cells, and appearance of testicular structure at birth (Biason-Lauber *et al.*, 2004; Chassot *et al.*, 2008b; Jeays-Ward *et al.*, 2003; Parma *et al.*, 2006; Tomizuka *et al.*, 2008; Vainio *et al.*, 1999; Yao *et al.*, 2004). The shared phenotypes of the *Rspo1* and *Wnt4* knockout ovaries indicate that these two factors are components of a common pathway in the fetal ovary (see below). Genetic analyses further revealed that *RSPO1* is responsible for stimulating the expression of *Wnt4* (Fig. 7.4) (Chassot *et al.*, 2008b; Trautmann *et al.*, 2008; Yao *et al.*, 2004). The *RSPO1*/*WNT4* pathway apparently operates independent of *FOXL2* as the expression of these genes is not affected when either one of these genes become inactive (Chassot *et al.*, 2008b; Ottolenghi *et al.*, 2007).

RSPO1 and *WNT4* elicit their actions in ovarian somatic cells via β -catenin, the intracellular regulator of the canonical *WNT* pathway. Inactivation of β -catenin specifically in the steroidogenic factor 1 (SF1)-positive ovarian somatic cells (putative precursors of granulosa cells) produces ovarian defects similar to those found in *Rspo1* and *Wnt4* knockouts (Liu *et al.*, 2009; Manuylov *et al.*, 2008). The involvement of β -catenin is further confirmed by the gain-of-function experiments where ectopic activation of β -catenin in the absence of *Rspo1* or *Wnt4* restores normal ovarian development (Chassot *et al.*, 2008b; Liu *et al.*, 2010). These experiments also reveal a molecular connection between *RSPO1* and *WNT4* (Fig. 7.4). In the absence of β -catenin, *Rspo1* expression in the ovary remains unchanged, whereas expression of *Wnt4* is lost, indicating the requirement of *RSPO1* and β -catenin for *Wnt4* expression (Liu *et al.*, 2009). *RSPO1* is able to induce β -catenin either directly by itself or synergistically in the presence of *WNT* ligands *in vitro* (Binnerts *et al.*, 2007; Kim *et al.*, 2008, 2006; Lu *et al.*, 2008; Wei *et al.*, 2007). It remains to be determined whether *RSPO1* and *WNT4* act in a linear fashion or synergistically in activating β -catenin in the somatic cells of the ovary.

Despite their different modes of action (secreted factor versus transcription factor), *RSPO1*, *WNT4*, and *FOXL2* eventually converge for a common purpose: maintenance of the identity of granulosa cells. In the absence of *Rspo1*, *Wnt4*, or *Foxl2*, the XX gonadal primordia develop into ovaries initially but testis components (Sertoli cells and testis cords) start to appear amongst ovarian structure after birth (Chassot *et al.*, 2008b; Ottolenghi *et al.*, 2005; Tomizuka *et al.*, 2008; Vainio *et al.*, 1999). However, when *Wnt4* and *Foxl2* are inactivated together, *Sox9*, the testis-determining gene downstream of *SRY* (see Chapter 2 on testis development), is significantly upregulated, leading to ovary-to-testis sex reversal (Ottolenghi *et al.*, 2007). These observations support the model that extracellular (*RSPO1*/*WNT4*) and intracellular (*FOXL2*) factors synergistically direct the gonadal somatic cells to follow the ovarian path by antagonizing *Sox9* expression (Figs. 7.4 and 7.5). *Sox9* is expressed in gonads of both sexes before the onset of sex determination. *SRY* in the testis maintains/stimulates *Sox9* expression, whereas in the ovary *Sox9* expression is lost (see Chapter 2 on testis development). Lack of *Sry* and its downstream effectors in the ovary is thought to be responsible for the absence of *Sox9* expression. However, the rise of *Sox9* in the *Wnt4*/*Foxl2* double knockout ovary without the presence of *Sry* gene argues against this

notion. We hypothesize that if neither *Sry* nor RSPO1/WNT4/FOXL2 is present, the default status of gonads is testis, as a result of gradual increase of *Sox9* expression (Fig. 7.5). In the XY individual, SRY in the testis jump-starts *Sox9* expression, which subsequently suppresses the pro-ovary functions of RSPO1/WNT4/FOXL2. On the other hand, in the absence of *Sry* as in the XX individual, RSPO1/WNT4/FOXL2 prevents the rise of *Sox9* and its ability to induce testis differentiation, therefore, allowing the somatic progenitors to differentiate into granulosa cells and subsequent ovary organogenesis.

5. Emerging Pathways for the Establishment of Female Somatic Environment and Maintenance of Female Germ Cells

In addition to their involvement in ovarian somatic cell differentiation, RSPO1 and WNT4 have unique functions in maintaining proper ovarian environment and survival of female germ cells. One of the earliest morphological differences between fetal testis and ovary is the establishment of an organized vascular network in the testis and the lack of a similar structure in the ovary (Brennan *et al.*, 2002). When either *Rspo1* or *Wnt4* or follistatin (*Fst*) is inactivated, the testis-specific vasculature appears in the fetal ovary at 12.5 dpc (Jeays-Ward *et al.*, 2003; Tomizuka *et al.*, 2008; Yao *et al.*, 2004). In addition to this phenotype, female germ cells undergo apoptosis starting at 15.5 dpc and are lost at the time of birth (Tomizuka *et al.*, 2008a; Vainio *et al.*, 1999; Yao *et al.*, 2004). Similar vasculature and germ cell loss phenotypes are also observed in the fetal ovary that lacks β -catenin in the SF1-positive somatic cells (Liu *et al.*, 2009; Manuylov *et al.*, 2008).

The connection between *Rspo1*, *Wnt4*, β -catenin, and *Fst* is further confirmed by the genetic models in which the constitutively active form of β -catenin is introduced to the somatic cells of *Rspo1* or *Wnt4* knockout ovary. In the presence of the active β -catenin, normal ovarian characteristics are restored despite a lack of *Rspo1* or *Wnt4* (Chassot *et al.*, 2008b; Liu *et al.*, 2010). In the *Wnt4* knockout ovary, where *Fst* expression is lost, active β -catenin is able to maintain *Fst* expression, placing β -catenin downstream of *Wnt4* (Fig. 7.4). β -catenin probably stimulates *Fst* expression directly via the TCF/LEF consensus sequence in the promoter region of *Fst* (de Groot *et al.*, 2000; Willert *et al.*, 2002).

Fst, a component in the RSPO1/WNT4/ β -catenin pathway, encodes a secreted protein that antagonizes the activity of activins. Binding of FST to activins inhibits or limits the ability of activins to interact with their receptors, therefore, silencing the functions of activins (Muttukrishna *et al.*, 2004). Activins consist of either homodimers or heterodimers of activin β A and β B subunits. mRNA for activin β B (*Acbb*), but not activin β A, is present in the fetal mouse ovary although its expression is low (Yao *et al.*, 2006). *Acbb* mRNA expression is suppressed by RSPO1, WNT4, and β -catenin, and when any of these three genes is inactivated, expression of *Acbb* is significantly elevated (Yao *et al.*, 2006). The inhibitory effects of RSPO1/ WNT4/ β -catenin on *Acbb* expression are further confirmed by the finding that addition of active β -catenin to the *Wnt4* knockout ovary suppresses *Acbb* expression to the low levels seen in the normal ovary (Liu *et al.*, 2010).

The elevated *Acbb* in the *Rspo1*, *Wnt4*, and β -catenin knockout ovary leads to the hypothesis that *Acbb* could be responsible for appearance of testis-specific vasculature and loss of female germ cells. Two observations support this hypothesis: first, presence of exogenous activin B (protein product of *Acbb*) induces formation of ectopic testis vasculature in normal fetal ovaries in culture (Yao *et al.*, 2006) and second, when the *Acbb* gene is inactivated in the *Wnt4* knockout ovary, normal ovarian development is restored (lack of testis-specific vasculature and maintenance of female germ cells) (Liu *et al.*, 2010; Yao *et al.*, 2006). Intriguingly, inactivation of *Acbb* in the *Fst* knockout ovary also restores normal ovarian development (Yao *et al.*, 2006). These results support the model that in the

fetal ovary, *Wnt4* and *Fst* antagonize functions of *Acbb*. WNT4, via the action of β -catenin, suppresses but does not completely abolish the expression of *Acbb*. The function of FST is to antagonize and inhibit the action of the residual activin B to prevent it from inducing testicular vasculature and demise of female germ cells (Fig. 7.4).

When the ovary-specific functions of *Wnt4* was first discovered in 1999, *Wnt4* was labeled as the inhibitor that prevents the appearance of Leydig cells, the androgen-producing cells present only in the testis (Vainio *et al.*, 1999). In the *Wnt4* knockout ovary, androgen-producing cells appeared ectopically, leading to masculinization of the female. However, the identity of these ectopic androgen-producing cells was later found to be adrenal origin instead of Leydig cells (Heikkila *et al.*, 2002; Jeays-Ward *et al.*, 2003). In addition, these adrenal cells appeared in not only the fetal ovary, but also fetal testis of the *Wnt4* knockout embryos (Heikkila *et al.*, 2002). The appearance of these androgen-producing adrenal cells are also observed in the *Rspo1* and β -catenin knockout ovaries (Chassot *et al.*, 2008a; b; Liu *et al.*, 2009; Manuylov *et al.*, 2008; Tomizuka *et al.*, 2008; Vainio *et al.*, 1999). Adrenals and gonads are derived from a common adrenogonadal primordium, which later separate into two identities. Presence of *Rspo1* and *Wnt4* in the adrenogonadal primordium before the separation of adrenal and gonads suggests that these two genes play a role in proper allocation of the adrenal cell lineage (Heikkila *et al.*, 2002; Jeays-Ward *et al.*, 2003). *Rspo1* and *Wnt4* probably are not involved in suppressing the appearance of Leydig cells in the fetal ovary.

6. Conclusion and Perspectives

Default or not, the pathways that lead to organogenesis of the ovary are far more complicated than what was originally hypothesized (Figs. 7.1 and 7.5). Components of the pathway possess properties of both ovary-determining gene and anti-testis Z factor, which operate at levels of cell-to-cell interaction and transcriptional regulation. Through the action of the secreted factors RSPO1 and WNT4, somatic cells in the fetal mouse ovary are instructed to follow the program for granulosa cell differentiation. Signals from RSPO1 and WNT4 are interpreted intracellularly via β -catenin, which with a synergistic action of FOXL2, silences the SOX9-induced testis differentiation (Figs. 7.4 and 7.5). This antagonism is a critical connection between ovarian somatic cells and germ cells. Lack of SOX9-induced testis differentiation results in absence of CYP26b1 (a putative target of SOX9) that degrades the meiosis-inducing RA. RA, therefore, becomes available only in the fetal ovary and induces meiosis of female germ cells. Once female germ cells have entered meiosis, they can only survive in the ovarian somatic environment that is set up by RSPO1, WNT4, and other components of the pathway (Fig. 7.4).

Despite this progress in our understanding of ovary organogenesis and its regulation, many questions remain. First, in addition to regulating somatic cell development, do *Rspo1* and *Wnt4* have a direct role on female germ cells? Female germ cells are almost completely lost in *Rspo1*, *Wnt4*, and β -catenin knockout ovaries at the time of birth. However, it is unclear whether the affected germ cells enter meiosis before their demise. Some reports show meiotic defects in female germ cells in the *Rspo1* and *Wnt4* knockout ovaries (Chassot *et al.*, 2008b; Naillat *et al.*, 2010). However, others find proper progression of germ cell meiosis in the absence of *Rspo1*, *Wnt4*, and β -catenin (Chassot *et al.*, 2008b; Tomizuka *et al.*, 2008; Liu *et al.*, 2009; Liu *et al.*, 2010). It is worth noting that the meiosis-inducing system (RA and Stra8) is not significantly altered in the *Rspo1* knockout ovary, but is compromised in the *Wnt4* knockout ovary (Chassot *et al.*, 2008b; Naillat *et al.*, 2010). More experiments are needed to reconcile these discrepancies and clarify whether RSPO1 and WNT4 intersect with RA in regulating germ cell meiosis.

Second, do fetal and adult ovaries utilize different mechanisms to maintain the differentiated state? Whereas loss of *Foxl2* does not affect formation of the fetal mouse ovary, *Foxl2* is essential for the maintenance of somatic cell identity in the adult ovary. Inactivation of *Foxl2* in the adult mouse ovary leads to upregulation of *Sox9*, transdifferentiation of granulosa cells into Sertoli cells, and appearance of testis structure and cell types (Uhlenhaut *et al.*, 2009). Granulosa-to-Sertoli transdifferentiation is also observed in adult ovaries of estrogen receptor alpha and beta double knockout (ER α β KO) and aromatase knockout mice (Britt *et al.*, 2001; Britt and Findlay, 2003; Britt *et al.*, 2004a, b; Dupont *et al.*, 2003, 2000). FOXL2 is known to stimulate expression of aromatase, the enzyme responsible for estrogen synthesis (Pannetier *et al.*, 2006; Uhlenhaut *et al.*, 2009; Wang *et al.*, 2007). It is therefore proposed that in the adult ovary FOXL2 serves to maintain granulosa cell identity by promoting the synthesis of estrogen, which is the conserved mechanism responsible for ovary formation in species such as reptiles, birds, fish, and even marsupials (Bruggeman *et al.*, 2002; Elf, 2003; Kobayashi and Nagahama, 2009; Mittwoch, 1998; Nakamura, 2009; Pask and Renfree, 2001; Yao, 2005; Yao and Capel, 2005). The maintenance of granulosa cell differentiation apparently shifts from estrogen-independent at the fetal stage to estrogen-dependent in adulthood (Fig. 7.6). Female mammals, particularly eutherian mammals, are constantly exposed to maternal estrogens. The estrogen-insensitive mechanisms for ovary organogenesis make physiological sense; otherwise all the male embryos would be sex-reversed by estrogens. Finding out what roles the RSPO1/WNT4 pathway plays in other vertebrate species will shed light onto the evolution of the mechanism for ovary organogenesis.

Third, where do the theca cell lineage originate from and how is their identity established? Theca cells, the female counterparts of testis Leydig cells, are essential for steroidogenesis and formation of the follicles. At present, no lineage markers have been identified for theca cells. Based on their similar functions and mesenchymal nature to Leydig cells, we propose that the mechanisms for their specification could also share similarities with Leydig cells. Specification of fetal Leydig cells is under the control of Sertoli cell-derived Desert hedgehog (Barsoum *et al.*, 2009; Barsoum and Yao, 2010; Huang and Yao, 2010; Yao *et al.*, 2002). We are currently investigating whether hedgehog ligands derived from the granulosa cells instruct mesenchymal precursor cells to differentiate into the theca cell lineage in the fetal ovary.

A final, important question is whether knowledge gained from mouse models is applicable to other mammalian species such as human. It is naïve to think that animals utilize identical regulatory pathways for one biological process such as ovary organogenesis. Components of the pathways may be conserved but species variations are expected as the organisms adapt to their unique developmental environment for survival. Comparing the mouse genetic models with human clinical cases collected so far (Table 7.1), *RSPO1* and *WNT4* both are involved in ovary organogenesis with species differences. Loss of function in human *RSPO1* gene leads to complete female-to-male sex reversal (Parma *et al.*, 2006), a phenotype much more severe than that in the mouse *Rspo1* knockout model (Chassot *et al.*, 2008b; Tomizuka *et al.*, 2008). Human patients with defective function of *WNT4* develop various degrees of female-to-male sex reversal (Biaison-Laubert *et al.*, 2004; Mandel *et al.*, 2008). In addition, humans seem to be more sensitive to gene dosage than the mouse (see gain-of-function cases in Table 7.1).

Despite these species variations, research using mouse models has identified or confirmed the involvement of components in the pathways toward organogenesis of the ovary. New candidate genes continue to be discovered by mRNA array experiments (Beverdam and Koopman, 2006; Bouma *et al.*, 2009; Cederroth *et al.*, 2007; Coveney *et al.*, 2008; Houmard *et al.*, 2009; Jorgensen and Gao, 2005; Nef *et al.*, 2005), protein screening (Ewen *et al.*,

2009), or regulatory sequence comparison (Lee *et al.*, 2009). It will not be a surprise in the near future if components of ovary organogenesis expand from X, Y, and Z to the entire alphabet.

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Figure 7.1.

Evolution of the hypotheses for sex determination in eutherian mammals. In 1972, Jost extended his paradigm on sexually dimorphic development of the reproductive tracts to gonad differentiation and proposed that a putative “male organizer” prevents the gonadal primordium from developing into an ovary and forces it to become a testis. In 1983, Eicher and Washburn proposed that an ovary-determining gene (*Od*) initiates ovary differentiation in the XX individual. The testis-determining gene on the Y chromosome (or *Tdy*), which becomes active earlier than the *Od* gene, suppresses the *Od* gene in the XY individuals. The Z theory was proposed in 1993 by McElreavey *et al.* after the discovery of *SRY* gene. It was stated that the Z gene in the XX gonad inhibits the testis pathway, therefore leading to the

progression of the ovary pathway. In the XY gonad, the *SRY* acts as an inhibitor of the Z gene, allowing the development of the testis.

Figure 7.2.

Morphogenesis of the mouse fetal ovary. Once the PGCs migrate into and colonize the genital ridge, they coalesce with somatic cells which could be derived from three potential sources: ① the neighboring mesonephros, ② the existing mesenchyme, and/or ③ the surrounding coelomic epithelium. (A) Somatic cells and PGCs form ovigerous cords. Female germ cells start to enter meiosis around 13.5 dpc. (B) Germ cell nests that are surrounded loosely by somatic cells begin to form. (C) Around the time of birth, somatic cells start to break down the germ cell nests by enclosing individual oocytes. (D) Breakdown of the germ cell cysts leads to the formation of the primordial follicles.

Figure 7.3.

Regulation of germ cell entry into meiosis in the developing gonads. (A) At 11.5 dpc, PGCs are present in the genital ridge and RA is produced in the neighboring mesonephric duct and tubules. *Cyp26b1* is expressed at low levels in the gonad of both sexes. The mesonephric tubules, which produce RA, are physically connected with the anterior (Ant) end of the gonad during this time. (B) In the male gonad, once *Sry* is expressed (~11.5 dpc), the RA-degrading enzyme *Cyp26b1* expression is upregulated. The testis cords, which form around germ cell clusters around 12.5 dpc, might concentrate the enzyme in these regions, thereby protecting germ cells from the actions of RA. Germ cells in the male gonad therefore do not enter meiosis at 13.5 dpc. (C) In the female gonad, *Cyp26b1* expression is detectable at 11.5 dpc, but disappears by 12.5 dpc. Germ cells at the anterior end of the gonad begin to express *Stra8* at 12.5 dpc. By 13.5 dpc, female germ cells enter meiosis in an anterior-to-posterior (Post) wave. Germ cells at the anterior end of the gonad might be exposed to RA earlier than those at the posterior end, or the RA concentration might be greater at the anterior end than the posterior end [this figure is modified from Figure 2 in Bowles and Koopman (2007)].

Figure 7.4.

Putative pathways for ovary organogenesis in the mouse embryo. (A) Two somatic cell-derived factors, R-spondin1 (RSPO1) and WNT4, activate synergistically or independently the canonical β -catenin (β -cat) pathway in XX somatic cells in an autocrine or paracrine manner. (B) β -catenin then induces expression of *Wnt4* while suppresses expression of *Sox9* (probably through the action of FOXL2) and its putative downstream target *Cyp26b1*. Without the presence of *Cyp26b1*, RA is not degraded and therefore induces *Stras8* expression and meiosis in DAZL-positive germ cells. (C) β -catenin also induces expression of follistatin (*Fst*) and at the same time maintains a low expression of activin β B (*Acbb*). FST antagonizes the action of activin B, which is the protein product of *Acbb*. Lack of activin B ensures that no testis-specific vasculature forms and the survival of female germ cells is maintained. See text for more details. (See Color Insert.)

Figure 7.5.

The 2010 version of sex determination hypothesis based on mouse genetic models. We propose that if both *SRY* and *RSPO1/WNT4/FOXL2* are absent, the default status of gonads is testis, as a result of gradual increase of *Sox9* expression. When both *SRY* and *RSPO1/WNT4/FOXL2* are present as in the XY individual, *SRY* in the testis jumpstarts *Sox9* expression, which subsequently suppresses the pro-ovary functions of *RSPO1/WNT4/FOXL2*. On the other hand, in the absence of *Sry* as in the XX individual, *RSPO1/WNT4/FOXL2* prevent the rise of *Sox9* and its ability to induce testis differentiation, therefore allowing the gonads to follow the ovarian path. When *Sry* is nonfunctional or lost, *RSPO1/WNT4/FOXL2* synergistically suppresses *Sox9* expression and facilitates ovary organogenesis. If the action of *RSPO1/WNT4/FOXL2* is silenced in the XX individual, the default testis pathway arises despite the absence of *Sry*.

Figure 7.6.

Maintenance of granulosa cell identity in fetal and adult ovaries. In the fetal ovary, granulosa cell identity is maintained in an estrogen-independent manner via the RSPO1/WNT4/β-catenin pathway and FOXL2, which together repress the expression of Sox9. This mechanism becomes estrogen-dependent after birth and in the adult ovary FOXL2 stimulates estrogen-producing enzyme aromatase and through the action of estrogen (E₂) and their receptors (ERα/β) suppresses Sox9 and maintain the identity of granulosa cells.

Table 7.1

Genes involved in fetal ovary development in mice and humans

Gene	Mutation	Sex	Phenotypes in mice	Phenotypes in humans	Reference
<i>Cttnb1</i> *	LOF*	F	Appearance of testis-specific vasculature, loss of female germ cells, and appearance of androgen-producing adrenal cells	None reported	Liu et al., 2009; Manuylov et al., 2008.
<i>Cttnb1</i>	GOF*	M	Testis-to-ovary sex reversal	None reported	Maatouk et al., 2008.
<i>Dax1</i>	LOF	F	No phenotypes in the fetal ovary	X-linked syndrome with adrenal hypoplasia congenital and hypogonadotropic hypogonadism	Meek et al., 2003a; Yu et al., 1998; Zanaria et al., 1994.
<i>Dax1</i>	GOF	M	Insertion of multiple copies of <i>Dax1</i> gene in the XY mouse delays testis development and causes male-to-female sex reversal in the <i>Y^{Pos}</i> background	Duplication of a portion of the X chromosome containing <i>DAX1</i> gene cause male-to-female sex reversal	Barbaro, 2008; Sanlaville et al., 2004; Swain et al., 1998.
<i>Foxl2</i>	LOF	F	Defects in folliculogenesis postnatally and Blepharophimosis Posis Epicanthus inversus Syndrome (BPES)-like symptoms	Premature ovarian failure and Blepharophimosis Posis Epicanthus inversus Syndrome (BPES)	Crisponi et al., 2001; Ottolenghi et al., 2005; Schmidt et al., 2004.
<i>Fst</i>	LOF	F	Appearance of testis-specific vasculature and loss of female germ cells	None reported	Yao et al., 2004.
<i>Rspo1</i>	LOF	F	Appearance of testis-specific vasculature, loss of female germ cells, and appearance of androgen-producing adrenal cells	Female-to-male sex reversal; Palmoplantar hyperkeratosis (PPK) and predisposition to squamous, cell carcinoma of the skin (SCC)	Chassot et al., 2008a; Parma et al., 2006; Tomizuka et al., 2008.
<i>Wnt4</i>	LOF	F	Appearance of testis-specific vasculature, loss of female germ cells, and appearance of androgen-producing adrenal cells	Various degree of female-to- male sex reversal and SERKAL syndrome (sex reversal; dysgenesis of kidneys, adrenals, and lungs)	Biason-Laubier et al., 2004; Jeays-Ward et al., 2003; Mandel et al., 2008; Philibert et al., 2008; Sultan et al., 2009; Vainio et al., 1999.
<i>Wnt4</i>	GOF	M	Transgenic mouse carrying human <i>Wnt4</i> gene disrupts testicular vasculature and decreases testosterone synthesis	Duplication of a DNA fragment containing <i>WNT4</i> gene leads to XY female phenotypes	Jordan et al., 2001, 2003.

* *Cttnb1* = β -catenin; LOF = loss of function; GOF = gain of function