THE ROLE OF BETA-CATENIN IN THE DEVELOPMENT OF FETAL OVARY AND FEMALE GERM CELLS

BY

CHIA-FENG LIU

DISSEPTION

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Doctoral Committee:

Associate Professor Humphrey H.-C. Yao, Chair
Professor Indrani C. Bagchi
Professor Janice M. Bahr
Associate Professor David Bunick
Assistant Professor Joan S. Jorgensen, University of Wisconsin-Madison
Ovarian development in mammals is considered as a default process, arising only in the absence of Sry, a testis-determining gene. However, current evidence has suggested the presence of an active pathway in fetal ovary. In particular, gene screening experiments have shown that many genes are expressed specifically in ovary. Wingless related protein (WNT) proteins are one class among them. WNT proteins are known to be essential for embryo development in many species. Multiple WNT ligands, including the ones that signal via the canonical β-catenin pathway, are expressed in fetal gonads, suggesting their intimate association with β-catenin and potential role in regulating ovary development. Thus, the central hypothesis of my studies is that the canonical β-catenin signaling pathway is essential for the development of fetal ovary.

Specifically, I conducted the studies to 1) determine the functional roles of β-catenin in the ovarian development, 2) identify the causes of female germ cell loss in the absence of WNT4/β-catenin signaling pathway, and 3) investigate the molecular connection between Wnt4 and β-catenin.

In the first study of determining the functional roles of β-catenin, I generated a transgenic mouse with β-catenin being ablated specifically in the steroidogenic factor 1 (SF1)-positive population of somatic cells of the fetal gonads. In the absence of β-catenin, fetal testis developed normally whereas unique phenotypes appeared during the development of ovary. These phenotypes included formation of testis-specific coelomic vessel, appearance of androgen-producing adrenal-like cells, and the loss of female germ cells. The observed phenotypes were similar to those found in the R-spondin1 (Rspo1) and Wnt4 knockout (KO) ovaries, indicating that these three factors were in the same pathway to regulate ovary development. In addition, the expression of Wnt4 was abolished whereas the expression of Rspo1 was unaltered, which placed β-catenin as a mediator between RSPO1 and WNT4.

My second study determined what caused female germ cell loss in the absence of WNT4/β-catenin signaling pathway. The observations of the ectopic androgen production and the upregulated inhibin β b (Inhbb) expression were also found, along with germ cell loss, in the Wnt4 KO and β-catenin conditional KO (cKO) ovaries suggesting these two to be the potential causes of female germ cell loss. Female germ cells were still lost in the β-catenin cKO embryos after treating with flutamide, an anti-androgen drug. This result ruled out the ectopic androgen
production from being responsible for germ cell loss. On the other hand, the number of germ cells was restored in the Wnt4 and Inhbb double KO indicating that Inhbb was the cause of female germ cell loss in the absence of WNT4/β-catenin pathway.

In my third study, I investigated the molecular connection between Wnt4 and β-catenin. I demonstrated that activation of the stabilized form of β-catenin prevented the appearance of male-specific characteristics during the ovarian development and also supported the survival of female germ cells. The results indicated that β-catenin served in the downstream of WNT4 in the somatic cells of fetal ovary to regulate fetal ovary and female germ cell developments.

In summary, WNT4/β-catenin pathway is essential for the maintenance of ovarian identities and the survival of female germ cells. WNT4 acts on the SF1-positive somatic cells to activate β-catenin. The activated β-catenin prevents the male-specific phenotypes from appearing in the mouse ovary. The survival of female germ cells hinges upon a delicate balance between the somatic cell-derived factors: WNT4 maintains the survival of female germ cells by suppressing Inhbb expression in the somatic cells via β-catenin.
To my friends, my parents and "my family"
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Chapter 1: Literature Review of Fetal Ovary and Germ Cell Development

The ovary is the female sex organ and has two major functions. First, it produces gametes that are essential for propagation of species. Second, it produces sex hormones that maintain the homeostasis of the reproductive system as well as the bone and cardiovascular systems.

Defects in ovarian development cause serious medical problems such as infertility and cancer in human. Although defects in the fetal ovary are usually not fatal, they may have long-term effects on the affected individuals. Thus, it is essential to understand the underlying mechanisms that determine the cellular fate of the ovary during embryogenesis to understand the diseases of the ovary in humans. Experimental evidence has shown that although the regulation mechanism of gonadal development varies across the mammalian species, the genes involved in this regulation are similar. In addition, the mouse mutant is an excellent and valuable tool for gene function studies. Therefore I used mouse model to study the development of fetal ovary.

1.1 Formation of the gonadal primordium in mouse

The gonadal primordium, the progenitor of somatic cells of gonad, starts as a thickening of the epithelial layers that line the coelomic cavity around 10 days post coitum (dpc) in the mouse. (1-3). Several genes are essential for the establishment of the gonadal primordium, the undifferentiated testis or ovary. These genes are Empty spiracles homolog 2 (Emx2) (4), LIM homeobox protein 9 (Lhx9) (5), (6), Chromobox homologue 2 (Chx, or M33) (7), Steroidogenic factor 1 (Sf1) (8), (9) and Wilms tumor 1 homolog (WT1) (10), (11). These genes are expressed in the somatic cells and trigger the process of formation of the gonad. Without the expression of theses genes the gonad either degenerates or undergoes apoptosis. In addition, the gonadal phenotypes in these null mutations are similar in both sexes, indicating these genes work synergistically and hierarchically in a common pathway way to regulate the formation of the presumption gonads. Up to date, the genetic and biochemical studies have revealed that there is a complex interaction among these genes in regulating differentiation of indifferent gonad. It has been shown that LHX9 binds directly to the Sf1 promoter and cooperates with one of the isoform of WT-1, which lacks lysine-threonine-serine (-KTS) amino acid between zinc fingers three and four, to regulate transcription of Sf1 (12). In the cell culture system, it also was demonstrated that WT1 (-KTS) activates the expression of Sf1 (12). These results indicate that the LHX9 and WT-1 act upstream of Sf1 and synergize to regulate Sf1 expression during the formation of the
1.2 Current knowledge of sex determination

The genetic sex in most mammals, at least in eutherian, is determined at the time of fertilization. From animal studies and human cases, it has been shown that the presence of the Y chromosome plays a critical role in determining sex in eutherian. On the other hand, sex determination does not depend on X chromosome or its dosage. For examples, patients with Klinerfelter’s syndrome (XXY) develop testes despite the fact that they carry two X chromosomes whereas patients with Turner’s syndrome (XO) all have ovaries (13, 14). Gonad in both sexes starts as a bipotential organ, which means that it has ability to differentiate into a testis or an ovary. The outcomes of a bipotential gonad are determined by a Y-linked gene, SRY (Sex-determining region of the Y chromosome) (15), which functions as a DNA-binding protein to activate Sry-related HMG-box gene 9 (Sox9) and to control cellular events that are important for testicular development, including the proliferation of coelomic epithelium, the formation of blood vessels at the coelomic surface, the initiation of Sertoli cell differentiation and other cascades of cellular events (16-20). When Sry or Sox9 is mutated or absent, the bipotential gonad develops into an ovary (16). Thus, ovarian development is considered as a “default” pathway in the traditional view of gonadal development. However, this classical view of gonadal development has been challenged by other alternative theories after the discoveries of the formation of testes in XX individuals without the presence of Sry gene (21-24). Before the discovery of Sry gene, Eicher and Washburn observed the XY sex reversal in mice and proposed an alternative theory of sex determination. They hypothesized that an ovary-determining gene (Od), located on either the X chromosome or an autosome, drives ovarian development. On the other hand, a testis-determining gene on the Y chromosome (Tdy) is needed to inactivate the function of Od to initiate the male pathway (Eicher and Washburn, 1983). After the discovery of Sry gene, McElreavey and colleagues proposed a similar but modified hypothesis named “Z theory” according to the analysis of sex-reversal XX humans that occur without the presence of Sry. They stated that the ovary-specific Z factor(s) functioned as an inhibitor of the testis pathway thus allowing the initiation of the ovarian pathway in XX individuals. In their model, Sry in XY individuals acted as an inhibitor of Z factor(s) to activate the testis pathway (25). Which theory is correct is still unclear. However, up to date, new evidence has emerged to
suggest that sex determination in mammals is regulated by much more complicated mechanisms (26, 27).

1.3 Current knowledge of fetal ovary development

1.3.1 Somatic cell lineage of fetal ovary

At the fetal stage, the somatic-cell lineages can generally be classified into two different cell types, the supporting cell lineage and the steroidogenic cell lineage, based on their future functional roles (28, 29). In the ovary, the supporting cell lineage becomes granulosa cells and the steroidogenic cell lineage becomes theca cells. Granulosa cells support the germ cells inside the developing follicle. Theca cells, located in the ovarian interstitium outside the basement membrane of ovarian follicle, produce androgen, which is ultimately converted to estradiol by granulosa cells (30, 31).

1.3.1.1 Granulosa cell

The origin of the granulosa cell lineage is still controversial but evidence has suggested three possible sources. First, it was proposed that the granulosa cell was derived from the pre-existing mesenchyme based on the observations of the human fetus that germ cells induce the formation of granulosa cell from the adjacent mesenchymal elements (32). However, further studies indicated that these mesenchymal cells were actually derived from the mesonephric region (33), which suggested that the origin of granulosa cell was from the mesonephric region of the rete ovarii (34). That the origin of granulosa cell is from the mesonephric region is further supported by the studies in mice and ovine. During embryogenesis in mice, it is noted that there is a connection between rete cells (mesospheric cells) and pre-granulosa cells (follicle cells) (35, 36). The morphology of the early pre-granulosa cells is indistinguishable from the morphology of the invading mesonephric cells (35, 37). Histological studies in the fetal sheep ovary also revealed that mesonephric cells migrate into the gonad to form the ovarian structures and to make contact with oocytes (33). These mesonephric-derived cells proliferate throughout fetal development to form the granulosa cell lineage (33).

In addition to these two potential sources, ovarian surface epithelium (OSE), derived from the coelomic epithelium, is also considered a possible origin of granulosa cells. In the sheep ovary, ovigerous cords, located in the ovarian cortex, are open to the surface epithelium between
day 38 and day 90 of gestation(3). Thus, it limits the source for recruitment of pre-granulosa cells from the epithelia cells. Furthermore, the number of pre-granulosa cells in the cords at the time of formation is fewer than 10% of the total number of granulosa cells present in the newly formed follicles. Between day 38 and day 90 of gestation, the cell types that are highly proliferated are oogonia and the mesothelial cells of the surface epithelium whereas the pre-granulosa cells are less or not proliferated (3). These observations provide the evidence that the increase in pre-granulosa cells within the ovarian cortex occurs via the recruitment of the highly proliferated cells from the surface epithelium at the open ends of the ovigerous cords (38). In addition, the coelomic epithelium is proven to be a source of Sertoli cells by using the cellular lineage tracking dye (1). Therefore, as the counterpart cell type of Sertoli cell in the ovary, granulosa cells can originate from the coelomic epithelium too. However, further study to confirm this hypothesis is desired. In humans, cell proliferation of cords in the ovarian cortex appears to be linked to the ingrowths of the proliferating superficial epithelium (39, 40). Thus, the OSE is believed to be part of the progenitor cells of granulosa cells in humans. According to the studies in different mammals, the origin of granulosa cells seems to be different from one mammalian species to another. However, most evidence supports the idea that both mesonephric region of the rete ovarii and surface epithelium contribute to granulosa cell lineage.

The differentiation of granulosa cells requires proper expression of a transcription factor, Foxl2. FOLX2, a somatic cell-specific factor, is expressed specifically in the pre-granulosa cells. Null mutation in Foxl2 results in the failure of the transformation of granulosa cells from squamous to cuboidal in the primordium follicle, which leads to arrest of folliculogenesis and early depletion of the follicular pool (41, 42). FOXL2 mutation in humans places these individuals at high risk of developing premature ovarian failures (43). This evidence indicates that Foxl2 plays an important role in regulating the development of granulosa cells. In addition to the somatic cell factor, a female germ cell specific factor, factor in germ line a (Figα), is essential for the recruitment of granulosa cells to form the primordial follicle (44). Figα is a transcription factor and is expressed specifically in female germ cells starting at 13.5 dpc. In the absence of Figα, primordial follicles do not form and massive degeneration of female germ cells occurs after birth (44). The previous observations also demonstrate that the interaction between somatic cell and germ cell is also critical for ovary development.
1.3.1.2 Theca cells

The origin of theca cells is believed to come from fibroblast-like precursors in the ovarian mesenchyme. This concept is due to the fact that theca cells are positioned outside the basement membrane of the ovarian follicle. Thus the origin of the theca cell is considered to be derived from and to remain within the mesenchymal compartment (30, 31). Using long-term continuous infusion of [3H]-thymidine to label newly proliferated cells and comparing the cellular structures, the precursor of theca cells is visualized in the ovarian stroma and interstitium at outset of the follicle growth (45). These results further confirm that the origin of the theca cell is from the ovarian mesenchyme.

Because theca cells are only present and adjacent to developing ovarian follicles, it seems that the differentiation of theca cells is closely associated with granulosa cells (46, 47). Magarelli and Magoffin demonstrated that the developing follicles secrete small-molecular-weight proteins, named thecal differentiating factors (TDFs), that stimulate thecal differentiation (48). Although the identity of TDFs is still unclear, at least one of these proteins is secreted under the control of follicle stimulating hormone (FSH) (48). In addition, studies using a bovine co-culture system indicated that cortical but not medullary stromal cells are actively transformed into theca cells with the presence of granulosa cells (46, 47). These evidences indicate that the differentiation of theca cells requires granulosa cells.

1.3.2 Molecular mechanisms involved in the development of the fetal ovary

Although ovarian development is thought to be a default process, which only emerges in the absence of the Y-chromosome or Sry gene, genetic screening and microarray analyses have identified many genes with ovary-specific expression such as Dax1 (49), Foxl2 (21, 50), follistatin (51, 52), Wnt4, (53), Wnt5a, Wnt6a, Wnt9a, (54), and R-spondin 1 (24, 55, 56). These genes are believed to be involved in ovarian development. Among them, Wnt4 and R-spondin1 (Rspo1) are of particular interest because some of their functional profiles match with some criteria of the ovarian determining gene and Z gene.

WNT4 and RSPO1 are paracrine and/or autocrine factors that support ovary development by inhibiting ectopic appearance of male-specific characteristics in females (24, 53, 55, 56). They are initially expressed in the somatic cells of both sexes but become female-specific after
the time of sex determination, i.e. 12.5 dpc in mouse (24, 53, 55, 56). Genetic analysis reveals that RSPO1 acts upstream of WNT4 (Chassot et al., 2008; Tomizuka et al., 2008). In addition, similar defects in reproduction system appear in the absence of Wnt4 and Rspo1 (24, 52, 53, 55-58), indicating these two factors form a cascade pathway to regulate fetal ovary development.

Null mutation in Rspo1 and Wnt4 does not affect the formation of the ovary but results in unique defects in the fetal ovary. First, a testis-specific vessel, coelomic vessel, appears in the Rspo1 and Wnt4 knockout (KO) ovary (52, 55, 58). The coelomic vessel is the landmark structure of early testis development and does not appear in the normal ovary. In addition to RSPO1 and WNT4, Follistin (FST) is another female-specific factor that plays a role in inhibiting formation of the testis-specific vasculature in the fetal ovary (52). Coelomic vessel also appears in the Fst null ovary (52). Epistatic studies reveal that Fst is in the downstream of Wnt4 (52). Genetic evidence further indicates that inhibin beta b (Inhbb), the subunit of activin B or inhibin B, is responsible for the formation of coelomic vessel in the absence of Rspo1 Wnt4, and Fst (55, 59). The expression of Inhbb is upregulated in the absence of Rspo1 and Wnt4 but not in the Fst. Nevertheless, the coelomic vessel does disappear in the double null mutation of Wnt4−/−; Inhbb−/− or Fst−/−; Inhbb−/−, indicating that Inhbb is responsible for the appearance of coelomic vessel in these KO ovaries (59). Inhibins usually do not activate signaling whereas activins trigger signaling cascades by binding to their receptors (60). Therefore, WNT4 and its related signaling cascades likely prevent the formation of testis-specific vessel by suppressing the transcription of Inhbb whereas FST functions as an activin binding protein that blocks the action of activin B from binding to its receptors (59). Because the formation of coelomic vessel is a testis-specific event, one would assume that this event should be controlled by Sry and its downstream signaling. However, the expression of Sox9, the putative downstream effector of Sry is not maintained or expressed in above female-specific null mutation ovaries (52, 55, 56, 61). Thus, the formation of coelomic vessel might be independent from Sry and its downstream signaling.

In addition to the formation of testis-specific vessel, another interesting phenomenon that is found in both Rspo1 and Wnt4 KO mice is the ectopic appearance of androgen-producing cells in the fetal ovary (53, 55, 56, 58). Because the function of androgen is to maintain the male reproductive tracts, it is necessary to inhibit the steroidogenic activity in the fetal ovary to
maintain the female identities. The steroidogenic cells appear in the fetal ovary in the absence of
Rspo1 and Wnt4 (53, 55, 56). These steroidogenic cells produce androgen, which results in the
appearances of epididymis and maintenance of male reproductive tract system in females (53,
55, 56, 58). Wnt4 was thought to inhibit the differentiation of Leydig cell in the fetal ovary (53).
However, these androgen-producing cells are not Leydig cells but are adrenal origin because of
the expression of adrenal markers, i.e. CYP21 (62). In addition, these cells are also present in the
Wnt4 null testis (62). Adrenal cells and Leydig cells are derived from the same primordium, the
adrenal-gonadal primordium. Wnt4 seems to control the proper separation of adrenal cell from
the gonadal primordium (58, 62).

The last interesting phenotype in the Rspo1 and Wnt4 null mutation mice is the loss of
female germ cell around birth. In the absence of these two paracrine factors from the somatic
cells, female germ cells enter meiosis properly but undergo apoptosis after 16.5 dpc and
eventually undergo massive depletion at birth (52, 55, 56). Follistatin a somatic cell factor, also
plays a role in maintaining meiotic germ cell survival in the fetal ovary (52). Female germ cells
are essential for the formation of ovarian follicle, which is the functional unit of ovary. Without
germ cells, the ovarian follicle will not form and the ovarian characteristics are lost. These
somatic cell-derived factors are not only essential for the survival of female germ cells but are
required for the maintenance of ovarian identity. The development of partial male traits in the
Wnt4 or Rspo1 null mutation mice suggests that Rspo1 and Wnt4 are involved only partly in the
ovarian development in mice and there may be other factors involved.

Dax1 gene (dosage-sensitive sex reversal, adrenal hypoplasia critical region, on
chromosome X, gene 1), an orphan nuclear receptor, is located on the X chromosome and is
thought to be a candidate for ovary determining factor according to the observation of the
duplicate of this gene in XY individuals causing the male-to-female sex reversal (63). It was
speculated that Dax1 might have function in the dosage-based sex-determining system. For
example, over-expression of Dax1 in XY mice caused sex reversal even Sry function was intact
(49). However, the loss-of-function studies showed that instead of playing the role in ovarian
development, Dax1 is critical for the development of testis (64-67). Therefore, it can be excluded
the involvement of Dax-1 in participating fetal ovary development in mouse.
Another potential factor that may affect the development of ovary is FOXL2. FOXL2 belongs to a forkhead transcription factor family and expresses specifically in ovary at the time of sex determination (22). This expression pattern is observed across different species including mouse, chicken turtles and fish, suggesting a conserved role of Foxl2 in the ovarian development in vertebrate (68, 69). Here I use the examples of human, goat, and mouse to elaborate the role of FOXL2 in ovary development.

FOX2L mutations in the humans lead to the BPES (Blepharophimosis Ptosis Epicanthus inversus Syndrome) and develop premature ovarian failure in females (22). However, BPES patients do not develop XX sex reversal. The phenotype is different from patients with RSPO1 and WNT4 mutations who may develop complete XX sex reversal or various degrees of XX sex reversal (24) (23, 57). In goats, FOXL2 has been link to PIS (Polled intersex syndrome), which is the case of XX to XY sex reversal in goat. In addition, the results of immunohistochemistry and RT-PCR analysis show that R-spondin1 and FOXL2 act into two distinct cellular types during ovarian development in goat (70). In mice, null mutation of Foxl2 affects the differentiation of granulosa cells. Follicles disappear in the Foxl2−/− ovary whereas the development of oocytes is not altered (42, 71). These examples demonstrate that FOXL2 plays different roles from RSPO1/WNT4 in ovarian development. Although FOXL2 and the RSPO1/WNT4 signaling pathway may act independently, there is evidence indicating that these two pathways act in a complementary manner in regulating ovary development (26). When Wnt4 and Foxl2 were both inactivated in the mouse fetal ovary, female-to-male sex reversal occurs and germ cell development is also affected (72). In addition, when either Wnt4 or Foxl2 is absent in the ovary, the expression of Sox9, a factor that promotes the testis program, is transiently expressed on 11.5 dpc in Wnt4−/− or upregulated perinatally in Foxl2−/− (73-75). Theses results indicate that WNT4 and FOXL2 may work synergistically to suppress the testis pathway, i.e. by inhibiting Sox9 expression, in the ovary and to establish the proper development of fetal mouse ovary.

RSPO1 and WNT4 are essential for the maintenance of ovarian identity during fetal stage in mouse whereas FOXL2 seems to be more critical in the adulthood. Conditional inactivated Foxl2 in the adult ovary leads to granulosa/theca cells undergo transdifferentiation into Sertol/Leydig-like cells and upregulation of testis-specific gene Sox9 in the follicle cells after puberty (Uhlenhaut et al., 2009). These phenotypes are similar to the ones that are observed
in the estrogen receptor alpha and beta double KO (ERαβKO) and aromatase knockout mice (76-78). In the absence of estrogen signaling, the ovary forms normally but it develops testis-specific tissues after puberty. Granulosa cells transdifferentiate into Sertoli cell and express Sox9. Seminiferous tubule-like structures appear and Leydig cells emerge in the interstitium (79-81). Although the ovarian development during fetal stage in mouse is apparently estrogen independent, the maintenance of ovarian identity in the adult still required estrogen signaling.

1.4 Formation and migration of primordial germ cells

Primordial germ cells (PGCs) are the progenitor cells of the germ cell lineage in both ovary and testis. PGCs are derived from the proximal epiblast adjacent to the extra-embryonic ectoderm. In mouse embryos as early as 6.25 dpc, the precursor of germ cells can be identified by the expression of B-lymphocytes-induced maturation protein 1 (Blimp1) in a founder population of epiblast cells (82). Blimp1 seems to be a critical determinant of the germ cell lineage in mice. In Blimp1 null mutation mice, around 20 primordial germ cell-like cells were found in the embryos but these cells failed to proliferate and migrate (82). Via interaction of several transforming growth factor beta (TGFβ) family members such as bone morphogenetic protein 4 (BMP4) and BMP8b form the extra-embryonic ectoderm, a cluster of PGC precursors arise from the proximal epiblast adjacent to the extra-embryonic ectoderm around 7.0 dpc in mouse embryos (83-86). By identifying the high level of alkaline phosphatase (ALP) activity, primordial germ cells are found to form a cluster of about 50 cells, posterior to the primitive streak at the base of the allantois by 7.2 dpc (87). PGCs then migrate through the hindgut and dorsal mesentery by 8.5 dpc and eventually enter the gonadal primordium by 10.5 dpc. On their way to the gonads, PGCs undergo mitotic proliferation and the number of germ cells increases from about 50 cells at 7.0 dpc to more than 25,000 cells by 13.5 dpc (88). Several factors have been identified to be essential for the survival of the PGCs during migration. These factors include Nanos3, Steel factor, a chemokine factor, SDF1 and FGFs. (89-93).

The expression of Nanos3 was detected around 7.5 pdc in the PGCs of mouse and maintained during the PGCs migration. In the absence of Nanos3, the number of apoptotic germ cells was increased whereas the proliferation rate was not affected during the migration (90). The increasing number of apoptotic germ cells during the migration resulted in few germ cells being present in the gonad (90). These evidence indicate that Nanos3 is essential for preventing PGCs from undergoing apoptosis during their migration.
Another factor that is essential for PGC’s survival and proliferation during their migration in the early mouse embryos is Steel factor, also known as kits ligand and Stem cell factor (89, 91, 94). The receptor of Steel factor, proto-oncogene receptor tyrosine kinase c-kit, expresses in the migrating germ cells starting from 7.25 dpc whereas the expression of Steel factor is in the neighboring cells along with the migrating germ (89, 91, 94). In the absence of either steel factor or its receptor, mice were sterile and reduced numbers of PGCs were seen during migration (95). In addition, the chemokine stromal cell-derived factor 1, SDF1/CXCL12, and its G-protein-coupled receptor, CXCR4, also played similar roles as Steel and c-kit in mouse germ cell migration and survival (92). FGFs (fibroblast growth factors) also are involved in migration of PGCs in mice (93). By using embryo slice culture, two FGFs and their receptors have been demonstrated to affect both motility and germ cell numbers. First, FGF2 and its receptor FGFR1IIIc on germ cells, regulated the motility of germ cells. Second, FGF7 and its receptors GFGR2IIIb on germ cells prompted the survival of germ cells during migration (93).

The interaction between the ligands from somatic tissues and their receptors on migrating germ cells indicated that the traveling of PGCs might be controlled by the surrounding tissue. After arriving at the genital ridge, the phenotypes of germ cells change. The shape of germ cells becomes more rounded and less motile. PGCs in male and female gonads are indistinguishable until days after they settle in the gonadal primordium. Once PGCs settle in the gonad, they are called gonocytes.

1.5 Current knowledge of female germ cell development
1.5.1 Meiotic regulation of female germ cells

Germ cells entering the XY gonad proliferate until 13.5 dpc and then undergo mitotic arrest. They resume proliferation after birth around 3 days of age. Male germ cells do not undergo meiosis until around puberty. However, when germ cells enter the XX gonad, they proliferate until 13.5 dpc and then undergo meiosis. Meiosis of female germ cells is the most obvious cellular event in the embryonic ovary. In the mouse fetal ovary, gonocytes enter the first meiosis between 13.5-14.5 dpc. They progress through leptonema, zygonema, pachynema and diplonema and then immediately arrest at the prophase of the first meiotic division (96). Establishment of sexually dimorphic development of germ cells has been disputed for many years. At least two mechanisms have been proposed for this divergent pattern. The first model
proposed that female germ cells enter meiosis following their intrinsic clock signaling. When germ cells are present in the ectopic region, such as mesonephros or adrenal glands (97-101), they still enter meiosis around 13.5 dpc, the same time as they do in the ovary. The alternative model is that the somatic cells of the fetal gonad provide the signals, which determine the fate of female germ cells. By culturing XY gonads using condition media from ovaries or testes with meiotic activity, Byskov et al. found that meiosis could be induced in the male germ cells. On the other hand, if XX gonads were cultured with fetal testes, the oocytes were prevented from reaching the diplotene stage of meiotic prophase. Thus Byskov and colleagues proposed that germ cell meiosis was controlled by the signals from by the somatic cells in the gonad: In the fetal ovary, the meiosis inducing substrate induces gonocytes to enter meiosis whereas, the meiosis-preventing substance from the testis prevents the meiosis of gonocytes (102-108). The resolution of the debate combined the elements of both the theories. Studies showed that retinoic acid (RA) influences germ cell meiosis by regulating the expression of Stra8 (stimulated by retinoic acid gene 8), which established the mechanism of sexually dimorphic development of germ cell meiosis. (109-113). CYP26B1, a P450 enzyme that is responsible for RA degradation, was undetectable in female gonads after 11.5 dpc but strongly expressed in the male gonads around 13.5 dpc in mice. RA signaling plays important roles in the development of different organ systems and its local concentration is controlled by balancing between synthesis and degradation (114, 115). The timing of Cyp26b1 expression was consistent with the initiation and entry of germ cell meiosis. In addition, when the RA signals from mesonephros in the XX gonad was blocked, the germ cell meiosis was not initiated. If the XY gonad was cultured with exogenous RA, the germ cells entered meiosis (116).

These experiments demonstrated the importance of RA in the initiation of germ cell meiosis and also supported that RA is a meiosis inducing substrate in the ovary. Furthermore, treatment of fetal testes with CYP26B1 inhibitors in culture induced the entry of XY germ cells into meiosis and expression of Stra8 in those cells (116, 117). Stra8 is required for premeiotic DNA replication and the subsequent events of meiotic prophase in germ cells of embryonic ovaries (117). STRA8 belongs to bHLH transcription factor and is expressed only in the XX germ cells before the initiation of meiosis during fetal stage (117). In the Stra8 null mutation mice, female germ cells failed to enter meiosis and thus the meiotic markers, such as Spo11 and dmc1 were undetectable at 14.5 dpc (117). In the presence of RA, the expression of Stra8 is
upregulated in the germ cell and therefore promoting the meiotic process of oocytes in the ovary. On the other hand, in the fetal testis, the presence of CYP26B1 enzyme degrades RA. The low level of RA resulted in the prevention of meiosis of XY germ cell in the testis. To respond the RA signal, female germ cells require the expression of an intrinsic factor, DAZL (Deleted in azoospermia-like), which is a germline specific RNA-binding protein, (118). In the absence of Dazl, germ cells could not respond to RA signals and in turn failed to enter meiosis (118). In addition, Nanos2, an RNA binding protein is specifically expressed in XY germ cell, also prevents the entry of XY germ cells into meiosis after the decrease of Cyp26b1 levels around 15.5 dpc (119). Null mutation of Nanos2 caused male gonocytes to enter meiosis and upregulated Stra8 expression (Suzuki and Saga, 2008). When Nanos2 was ectopically expressed in female germ cells, it resulted in the downregulation of Stra8 expression and also failure of meiosis initiation in those cells (119). Taken together, the meiosis of female germ cell is controlled by an extrinsic signal i.e. RA from mesonephros, as well as regulated by intrinsic signals, i.e. DAZL and Nanos2, from germ cells.

1.5.2 Regulation of the germ cell number in the fetal ovary

After reaching the peak number in mitotic proliferation during embryonic stage, the number of female germ cells decreases sharply particularly during two major time points, the prophase I of meiosis and the formation of primordial follicles (120-122). The apoptosis of female germ cells occurs in the ovary leaving a finite number of oocytes for the rest of the reproductive life of the female individuals (120, 121).

Two theories have been proposed for the death of female germ cells during embryogenesis (123, 124). The first hypothesis is that the intrinsic signal of oocytes causes the programmed cell death of abnormal oocytes. These abnormal oocytes may have defects on their chromosomes or mitochondrial genomes (125). This intrinsic pathway eliminates those abnormal oocytes to ensure the quality of oocytes in the ovary. For instance, most sex chromosome aneuploidies, e.g. Turner syndrome (XO), are often associated with gonadal dysgenesis with absence or near absence of germ cells in the gonad. Another theory is that the factors from the somatic cell environment control the fate of female germ cells. The shortage of those survival factors from the somatic cells may lead to death of female germ cells. For example, in the absence of Kit ligand from the somatic cell, germ cells degenerated via programmed cell death.
Loveland and Schlatt, 1997). In addition, the apoptosis of female germ cells mainly occurs in the medullary region of ovary during fetal stage (101). Therefore, it is possible that the cortex region of the ovary might provide a niche for female germ cells escaping death during the fetal stage (52). However, the underlying mechanism that is responsible for maintaining the appropriate number of oogonia is still unclear.

1.6 WNT/β-catenin pathway

The members of the WNT protein family are highly conserved glycoproteins with paracrine and/or autocrine functions. The crystal structure of WNTs has not been identified. However, it is known that all the WNT proteins share some common features including a signal sequence for secretion, several highly charged amino acid residues and many potential glycosylation sites. WNTs also contain a characteristic distribution of 22 cysteine residues, suggesting that proper protein folding might require the formation of multiple intra-molecular disulfide bonds (126). Wnt signaling pathway plays essential roles in the process of embryonic development, including organogenesis, cell fate decision, cell differentiation, segmentation and cell death (127-129). Wnt signaling pathway was first studied in detail in the Drosophila wingless genes and mammalian cancer research. The name was derived from the Drosophila gene, Wingless, and the related mammalian oncogene, Int-1. Up to date, there are nineteen Wnt genes in the human and mouse genome, seven in Drosophila, and five in C. elegans, indicating the conserved nature of this protein family in evolution. To elicit their functions, WNT proteins bind to their specific receptors, Frizzle and LRP5/6 (LDL-receptor related protein 5/6), on cell membrane and transduce their signals via at least three different intracellular pathways, the canonical Wnt/β-catenin pathway and the non-canonical Wnt/Ca^{2+} pathway and Wnt/Ras homolog gene family pathway, depending on the tissue context (130). In the canonical Wnt/β-catenin pathway, Wnt protein triggers its functions by activating its downstream key secondary messenger, β-catenin. Without the presence of Wnt protein, β-catenin is phosphorylated by a protein complex including GSK3β (glycogen synthase kinase 3β), CK1α (casein kinase 1α), APC (Adenomatous polyposis coli) and Axin, then degraded through the ubiquitin pathway (131). When Wnt ligand binds to the receptor and activates the intracellular signaling, GSK3β/Ck1/APC/Axin complex will be inhibited by disheveled (DVL) and thus leads to the stabilization of β-catenin. The stabilized β-catenin then translocates to the nucleus to form a
complex with T-cell factor/lymphoid enhancer factor (TCF/LEF) proteins, which results in the transcriptional regulation of downstream target genes of Wnt signaling \((132, 133)\). The simplified version of WNT pathway is shown in Fig. 1.

1.6.1 β-catenin

β-catenin is the member of the Armadillo (ARM) repeat protein superfamily. The structure of β-catenin contains a central stretch of 12 imperfect ARM repeats (R1-R12), a distinct N-terminal, carboxy-terminal domain that frames R1-R12, and a helix in the proximal carboxy-terminal domain, which is positioned against the last ARM repeat \((134)\). The molecular weight of β-catenin is about 86 kDa. The functional role of β-catenin was first discovered as a cell-to-cell adhesion molecule via cadherins and actin cytoskeleton, particularly in the epithelial cells \((135, 136)\). In the canonical Wnt pathway, β-catenin is a key mediator, which serves as a transactional co-regulator binding to specific DNA-binding transcription factors. Although several proteins, such as FOXO, PITX2, SOX9 and SOX17, are known to recruit β-catenin to target Wnts, most well-known transcriptional regulators that interact with β-catenin are T cell factor (TCF) and lymphoid enhancer factor (LEF). In the absence of this signaling, TCF acts as a repressor for Wnt target genes. When high level of β-catenin is present in the nucleus, it converts TCF from a transcriptional repressor to activator to trigger the transcription of target genes.

1.6.2 The involvement of WNTs in the fetal gonad

Based on the results from gene screening, several WNTs and their related proteins have been observed to be expressed in the fetal ovary \((54)\). However, only WNT4 and RSPO1 have been reported to be associated with sexual development \((24, 53, 55-57)\). Similar phenotypes, including the formation of testis-specific vessel, appearance of androgen-producing adrenal cells, masculinization of female embryos, and the loss of germ cell at birth, were observed in both Wnt4 and Rspo1 null mutation mice \((24, 53, 55-57)\). In the gain-of-function study, duplication of Wnt4 gene in mice caused the reduction of testosterone synthesis and disorganized the vascular system \((137)\). WNT4/RSPO1 pathway is apparently essential for the proper development of fetal ovary. Nevertheless, how do these two paracrine/autocrine factors elicit their functions? Evidence has shown that RSPO1 and WNT4 elicit their functions likely via β-catenin in other organogenesis studies \((56, 138-140)\). Based on promoter analysis studies, TCF/LEF consensus
sequence in the promoter region of Fst, a downstream target gene of WNT4, is a key cis-acting element for the expression of Fst (141, 142). Thus, WNT4 might regulate the expression of Fst through β-catenin expression in the ovary. In addition, overexpression of β-catenin in the male mice causes male-to-female sex reversal (27). According to the RT-PCR analysis, many WNT receptors are present in the ovary, which suggested that the WNT signaling pathway particularly, the canonical WNT/β-catenin pathway may play a role in the fetal ovary development. (143). Therefore, it is necessary to study the functional role of β-catenin in the fetal ovary development.

1.7 Specific aims

The first specific aim (Chapter 2) of this dissertation was to understand the involvement of β-catenin in fetal gonad development. I investigated whether β-catenin is a mediator of the RSPO1/WNT4 pathway in the process of fetal ovary development. To achieve the role of β-catenin in the RSPO1/WNT4 pathway, I inactivated β-catenin specifically in the somatic cells of the fetal gonad by using Cre/lox system. The results provided the genetic evidence to directly demonstrate that β-catenin played a role in the development of the fetal gonad. I also investigated the molecular interactions among β-catenin, Rspo1, and Wnt4 in the developing ovary.

The development of germ cells has been shown to be closely associated to their interactions with somatic cells. Female germ cells are essential for the organogenesis of the ovary; without them, formation of ovarian follicles is inhibited, which will result in the loss of the functional and structural characteristics of the ovary. One of the phenotypes in the absence of Rspo1/Wnt4/β-catenin signal is the loss of germ cells. Thus, my second specific aim (Chapter 3) was to investigate the causes of germ cell loss and the role of the β-catenin in maintaining the survival of germ cells. I first analyzed the development of female germ cells by using germ cell specific markers at different embryonic stages. I also examined whether female germ cells entered meiosis in the absence of β-catenin in the somatic cells of fetal ovary. In addition, I investigated the cause of germ cell loss in the absence of Wnt4/β-catenin signaling pathway by applying genetic or toxicological methods.

My third and last specific aim (Chapter 4) was to prove that β-catenin serves downstream of WNT4 to regulate fetal ovary and female germ cell development. To examine this hypothesis, I generated an animal model that constitutively activates β-catenin in the somatic
cells in the \textit{Wnt4} null mouse ovary. Furthermore, I applied molecular and immunoassay approaches to demonstrate the underlying molecular mechanisms.
Fig. 1 A simplified version of the canonical WNT/β-catenin pathway in mammals. (Left) In the absence of WNT ligand, its downstream target factor, β-catenin, will be phosphorylated by glycogen synthase kinase 3β (GSK-3). β-catenin then is targeted for degradation. (Right) in the presence of WNT proteins, WNT binds to the Fizzled/LRP receptor complex at the cell surface. These receptors transduce a cascade of cell signaling that prevent β-catenin from degradation. β-catenin accumulates in the cytoplasm and nucleus. β-catenin then interacts with TCF/LEF to control transcription of downstream target genes.
Chapter 2: Ablation of β-catenin in the Somatic Cells of the Fetal Ovary Causes the Loss of Ovarian Identity

2.1 Abstract

Sexually dimorphic development of the gonads is controlled by positive and negative regulators produced by somatic cells. Many Wnt ligands, including the one that signals via the canonical β-catenin pathway, are expressed in fetal gonads. β-catenin, a key transcriptional co-activator of the canonical WNT pathway and an element of the cell adhesion complex, is essential for various aspects of embryogenesis. To study the involvement of β-catenin in sex determination, a transgenic mouse with β-catenin ablated specifically in the steroidogenic factor 1 (SF1)-positive population of somatic cells was generated. Although β-catenin was present in gonads of both sexes, it was necessary for ovarian differentiation but dispensable for testis development. Loss of β-catenin in the fetal testis did not affect Sertoli cell differentiation, testis morphogenesis, or masculinization of the embryos. However, the molecular and morphological defects were observed in ovaries lacking β-catenin, including formation of testis-specific coelomic vessel, appearance of androgen-producing adrenal-like cells. These phenotypes were strikingly similar to those found in the R-spondin1 (Rspo1) and Wnt4 knockout ovaries. In the absence of β-catenin, the expression of Wnt4 was not detectable whereas that of Rspo1 was not changed, placing β-catenin as a component in between Rspo1 and Wnt4. These results demonstrated that β-catenin is responsible for transducing ovary-specific signals in the SF1-positive somatic cell population during mouse gonadal development.
2.2 Introduction

Gonadal primordium is the only organ rudiment that has the potential to differentiate into two different organs, an ovary or a testis. In most mammals, the decision of sex determination is triggered by the expression of Sry (sex-determining region of the Y chromosome), which leads to the activation of male-specific gene Sox9 (Sry-related HMG-box gene 9) and induces the cellular events that are important for testis morphogenesis (16-20). The transformation of the gonadal primordium into an ovary is thus considered to occur by default in the absence of Sry. However, the incidence of testis formation in XX individuals without the presence of Sry has led to the proposal of the “Z” hypothesis (21-25). The ovary-specific Z factor(s) acts as an inhibitor of the testis morphogenesis pathway, therefore allowing the emergence of the ovary pathway. Sry, instead of being a testis-inducer, antagonizes the Z factor to ensure the progression of the testis program. More evidence indicates that the level of antagonism is not restricted at the level of transcription. The testis and ovary both produce unique signaling molecules with opposing functions such as fibroblast growth factor 9 (Fgf9) and Wnt4, respectively (73). The outcome of the sex seems to hinge on the balance between the antagonizing pathways induced by these signaling molecules.

WNT4, a paracrine or autocrine molecule involved in one of the antagonizing pathways of sex determination, belongs to a highly conserved glycoprotein WNT family. Wnt4 null mice developed the partial female-to-male sex reversal with appearances of testis vasculature, ectopic androgen-producing cells, and the androgen-dependent Wolffian duct, the precursor of the male reproductive tract (52, 53, 58). WNT4 mutation in humans caused the different degrees of female-to-male sex reversal (23, 57). It has been shown that WNT4 could signal via β-catenin dependent or independent pathway depending on tissue context (52, 138, 139, 144-154). Studies on R-spondin1 null mice demonstrated that the genes involved in β-catenin signaling were down regulated in the fetal ovary. Null mutation in R-spondin1 caused partial female-to-male sex reversal, similar to the phenotypes that appeared in the Wnt4 KO mice (56, 144). These data implicate the possibility of the presence of the canonical WNT/β-catenin in the developing ovary to regulate ovary development. However, so far there is no direct evidence suggesting WNT/β-catenin is present and is regulating the gonadal development.
Wnt4 is expressed in both sexes on 9.5 days post coitum (9.5 dpc) and becomes ovarian specific at the time of sex determination around 11.5 dpc (53). However, in addition to Wnt4, there are other Wnts present in the gonads. Wnt5a, Wnt6 and Wnt9a are expressed in the ovary (155) whereas WNT1, WNT3, and WNT7A are present in the testis (156-158). The presence of multiple Wnts in the gonads suggests that WNT pathway might involve and control the gonadal development and most likely, might signal through β-catenin, the key mediator of the canonical WNT pathways as well as an important component of cell-cell adhesion.

Nonetheless, to date only the circumstantial evidence have been provided and to suggested that the canonical WNT/β-catenin pathway might be present and regulate the developing gonad. In this chapter, my goal is to understand the involvement of β-catenin in the sexual dimorphic development of fetal gonad. To determine the functional role of β-catenin during the development of the fetal gonads, the Cre/loxP system was performed to remove β-catenin specifically in the somatic cells during the gonadal development. The results reveal that β-catenin, as Rspo1 and Wnt4, is essential for maintaining ovarian identity but dispensable for testis formation.

2.3 Experimental procedures

2.3.1 Animals

Transgenic mice (SF-1/cre) carrying Cre recombinase under the control of the Steroidogenic factor 1 (SF-1) regulatory element (159) and floxed β-catenin mice (Ctnnb1^floxed/floxed, Jax#004152) (160) were maintained on a B6 background. To increase the efficiency of production of homozygous null alleles and to decrease the incidence of mosaic deletion, SF-1/cre;Ctnnb1^Fc embryos were generated. I followed the standard breeding scheme described by Kwan et al to generate the conditional β-catenin knockout mice (161). Four crossing steps were taken to generate the β-catenin conditional mice (cKO). The first cross was to mate homozygous floxed β-catenin male mice (Ctnnb1^floxed/floxed) with Ella-cre female to generate Ctnnb1^F-; Ella-cre/+ (Cross1, see crossing schemes below). The second cross was to get rid of Ella-cre allele by mating Ctnnb1^F-/; Ella-cre/+ with B6 mice to generate Ctnnb1^F- mice. Then the Ctnnb1^F- mice was mated with Sf1^cre/+ mice to generate Sf1^cre/+; Ctnnb1^F- (Cross3). Lastly, the Sf1^cre/+, Ctnnb1^F- was mated with Ctnnb1^floxed/floxed to generate β-catenin cKO mice (Cross 4).
The Cross schemes

Cross 1. \( Ctnnb1^{\text{floxed/floxed}} \) (male) \( \times \) Ella-cre (female) \( \rightarrow \) \( Ctnnb1^{+/+} \); Ella-cre/+

Cross 2. \( Ctnnb1^{+/+} \); Ella-cre/+ \( \times \) B6 \( \rightarrow \) \( Ctnnb1^{+/+} \)

Cross 3. \( Ctnnb1^{+/+} \) \( \times \) Sf1\(^{\text{cre/+}}\) \( \rightarrow \) Sf1\(^{\text{cre/+}}\); \( Ctnnb1^{+/+} \)

Cross 4. Sf1\(^{\text{cre/+}}\); \( Ctnnb1^{+/+} \) \( \times \) \( Ctnnb1^{\text{floxed/floxed}} \) \( \rightarrow \) Sf1\(^{\text{cre/+}}\); \( Ctnnb1^{\text{floxed/-}} \)

Female and male mice were housed together and checked for the presence of a vaginal plug the next morning. The day when the vaginal plug was detected was considered 0.5 days of gestation, or 0.5 days post coitum (0.5 dpc). Samples were collected at 12.5 dpc, 13.5 dpc, 15.5 dpc and birth (P0).

2.3.2 Genotyping

For genotyping, tail clippings were digested in 200 µl of 50 mM sodium hydroxide for 30-45 minutes at 95°C, then 100 µl of 1 M Tris HCl, pH=8 was added and mixed before centrifugation at 10,000 g for 5 minutes at room temperature. Samples were then subjected to standard PCR using gene-specific primers. A 25 µl standard PCR reaction was set up as following: 1 µl of tail DNA, 12.5 µl of 2X PCR Mango Mix (Bioline), 1.25 µl of 10 mM gene-specific primers and 10.25 µl of double distilled water. All PCR reactions were run using the BIO-RAD MyCycler Thermal Cycler followed by running on 1.5 % or 2% TAE gel at 100 volts. The primers and PCR reactions for genotyping were listed in Table 2.1 and Appendix A.

2.3.3 Histology

Tissues were fixed in 4% paraformaldehyde in PBS overnight at 4°C, processed through graded ethanol series and Histo-clean (International Diagnostics), and eventually embedded in paraffin. Five µm sections were obtained and rehydrated for hematoxylin and eosin staining (2 minutes for hematoxylin and 30 seconds for eosin). Slides were mounted using Permount.

2.3.4 Immunohistochemistry

Testes and ovaries were isolated and fixed in 4% paraformaldehyde overnight at 4 °C. The next day, samples were rinsed three times in PBS for 5 minutes each. Then samples were put through graded sucrose series (10%, 15%, and 20%) and incubated in 1:1 20% sucrose and CT
freezing media (Tissue-Tek) overnight at 4 °C. The samples were embedded in 1:3 20% sucrose and OCT mix and cut to 8-10 µm frozen sections. Sections were washed in PBS for 10 minutes and blocked in blocking solution (5% heat-inactivated donkey serum and 0.1% Triton X-100 in PBS) for 1 h at room temperature. Then sections were incubated with primary antibodies (see below) at 4 °C for overnight. The next day sections were washed three times for 10 minutes each with washing solution (1% heat inactivated donkey serum and 0.1% Triton X-100 in PBS) followed by incubation in the corresponding secondary antibodies. After secondary antibody treatment, sections were washed three times for 10 minutes each in washing solution and mounted with DAPI. The dilutions and sources of primary antibodies used were rabbit polyclonal antibody against β-catenin (1:500, Sigma), a goat polyclonal antibody against GATA-4 (1:250; Santa Cruz), a rat polyclonal antibody against anti-Müllerian hormone (AMH; 1:200; Santa Cruz), a rat monoclonal antibody against germ cell nuclear fraction Tra98 (1:1000; provided by H. Tanaka; (162), a rabbit polyclonal antibody against CYP17 (1:100; a gift from Buck Hales), a rat polyclonal antibody against PECAM-1 (1:500; Pharmingen), a rabbit polyclonal antibody against INSL3 (1:100; a gift from Stefan Hartung; (163), and a rabbit polyclonal antibody against SOX9 (1:1000; provided by Ken Morohashi (164). All the secondary antibodies (1:200) were fluorescein-conjugated from Jackson Immunochemical.

2.3.5 Transformation and mini preparation of plasmid

One µl of plasmid solution was added to 50 μl of DH5α competent cells in the tube and then placed on ice for 30 minutes. The tube was subjected to heat shocked at 42°C for 40 seconds. 1ml of Luria-broth (LB) or SOC media was added to the tube followed by incubation at 37°C for 1 hour. Bacteria solution was centrifuged at 2000 g for 5 minutes at room temperature. The 950 µl supernatant were discarded and bacteria were suspended in the remaining media (~100 µl) by pipeting. Next, 100 µl of the sample was placed on a Luria-broth ampicillin agar plate and incubated overnight at 37°C. The tip used to spot each of the colonies was dropped into a separate tube of 3 mL LB with ampicillin and incubated at 37°C with shaking for 16 hours. The Qiagen mini-prep kit was used for mini preparation (Cat. no. 27106, Qiagen, USA). In brief, the bacteria solution was collected in 1.5 ml tubes. The tubes were centrifuged at maximum speed for 1 minute and the supernatant was removed. Then, 250 µl of cold buffer P1 were added to the solution and the pellet was suspended using vortexing. Next, 250 µl of buffer P2 were added in
the tube and mixed with buffer P1 thoroughly by inverting the tube 4–6 times incubated at room temperature. Then 350 µl of buffer N3 were added and mixed immediately and thoroughly by inverting the tube 4–6 times. Samples then were centrifuged at 10,000 g for 10 minutes at room temperature and the supernatant was transferred to the QIAprep spin column following washing with buffer PB and buffer PE. Finally, DNA was eluted from the column by adding TE buffer.

2.3.6 RNA probe synthesis

Five µg of plasmid were treated with the appropriate restriction enzyme for linearization then purified by mixing with 1 volume Phenol/ Chloroform /Isoamyl alcohol (25:24:1) and centrifuging at 10,000 g for 10 minutes. Supernatant was moved to a new tube and the residual phenol was removed by one volume of chloroform/isooamyl alcohol (24:1). DNA was precipitated by adding 2 volumes of 100% Ethanol and incubated at -20 °C for at least 2 hours. DNA was then recovered by centrifugation at 10,000 g, 4 °C for 30 minutes and washed with ice-cold 70 % ethanol. The in vitro transcription reaction contained: 1 µg of linear plasmid, 2 µl of 10X transcription reaction buffer, 2 µl of Digoxigenin nucleotide mix, 1 µl of appropriate RNA Polymerase, and DEPC-water to a volume of 20 uL. Samples were incubated at 37°C for 2 hours. Then 0.8 µl of 0.5M EDTA was added to stop the reaction. Next, 1 µl of 10M Lithium chloride and 75 µl cold 100% ethanol were added to the reaction mixture. Samples were placed at -20°C for at least 2 hours followed by centrifugation at 10,000g speed for 30 minutes at 4°C. After the centrifugation, the supernatant was removed and the pellet was washed with ice cold 75% DEPC-ethanol and re-suspended in 20 µl DEPC water. Finally, the probe was diluted 1:1 with hybridization buffer (5x SSC pH 5.0, 50% formamide, 0.1% CHAPS, 0.1% Tween20, 1mg/ml Yeast tRNA, 50 µg/ml Heparin, and 5mM EDTA pH 8.0) and stored at -20 °C. A gel check was also utilized to determine if the probe was transcribed correctly.

2.3.7 Whole-mount in situ hybridization

Tissues were fixed overnight in 4% paraformaldehyde in PBS at 4°C and dehydrated through a methanol gradient (25%, 50%, 70%, and 100%) in PTW (0.1% Tween20 in DEPC-PBS). Samples were stored in 100% methanol at -20°C. In situ hybridization was processed according to the standard non-radioiostopic procedure using digoxigenin-labeled RNA probes. In brief, samples were dehydrated through a methanol gradient then treated with proteinase K (10
mg/ml) at 37°C for 12 minutes followed by fixation in 4% paraformaldehyde/0.1% glutaldehyde immediately at room temperature for 20 minutes. Samples were pre-hybridized in hybridization buffer (5x SSC pH 5.0, 50% formamide, 0.1% CHAPS, 0.1% Tween20, 1mg/ml yeast tRNA, 50 µg/ml heparin, and 5mM EDTA pH 8.0) at 65 °C for 1 hour. Then digoxigenin-labeled RNA probe was added into the solution and samples were rotated in an oven at 65°C overnight (12-16 hours). Samples were washed the next day in pre-warmed hybridization buffer, followed by MABTL (5% MAB, 0.1% Tween20 and 0.05% Levamisol). Samples were incubated in 20% sheep serum in MABTL blocking solution at room temperature for 2 hours followed by incubating in alkaline phosphatase-conjugated anti-digoxigenin at 4°C on shaker overnight. On the third day, after washed in MABTL three times for 1 hour each, samples were incubated in alkaline phosphates substrate in NTMTL (0.1 M NaCl, 0.01 M Tris-HCl pH 9.5, 0.05 M MgCl₂, 1% Tween20, 0.05% Levamisol) for color development. The optimal hybridization temperature and sources of RNA probes are: Rspo1 (65°C; Blanche Capel), Wnt4 (65 °C; Blanche Capel), Fst (65 °C; Martin Matzuk), Cyp21 (65 °C; Keith Parker), Sox9 (65 °C; Blanche Capel), and Fgf9 (60 °C; Blanche Capel).

2.4 Results

2.4.1 β-catenin was inactivated specifically in the somatic cells of fetal gonads

To investigate the role of β-catenin in the gonadal development, a β-catenin cKO mouse was generated. β-catenin was ablated in the somatic cells of fetal gonads by SF1/cre recombinase (159). The activity of the SF1/cre was detected in the somatic cells of gonads of both sexes as early as 11.5 dpc (159). A β-catenin antibody that has been shown to detect β-catenin in fetal mouse testis was used to confirm that β-catenin was removed specifically from the somatic cells of gonads (165). In the control testes (SF1/cre;CtnnbF/ or CtnnbF/C), β-catenin was detected on the membrane of AMH-positive Sertoli cells and germ cells starting at 15.5 dpc (Fig. 2.1 A & C, n=3). In contrast, in the β-catenin conditional knockout mice (SF1/cre;CtnnbF/C or β-catenin cKO, Fig. 2.1 B & D, n=3), the staining for β-catenin in Sertoli cells was abolished, whereas it was maintained in germ cells.

In control fetal ovaries as early as 12.5 dpc (Fig. 2.1 E & G, n=2), membrane β-catenin was detected in most somatic cells (PECAM-1 negative) and germ cells (PECAM-1 positive). In
the β-catenin cKO ovaries (Fig. 2.1 F & H, n=2), membrane staining of β-catenin was unaffected in PECAM-1-positive germ cells but disappeared in the PECAM-1-negative somatic cells. These results demonstrated that Cre recombinase directed by the SF1/cre transgene inactivated β-catenin in a somatic cell-specific manner.

2.4.2 Normal testis development in the absence of β-catenin

I first examined whether testes form properly in the absence of β-catenin in the somatic cells. SOX9 and AMH, two molecular markers for fetal Sertoli cells, were expressed in a manner indistinguishable between control and β-catenin cKO testes at 12.5 dpc (Fig. 2.2 A & B, n=3). Testis cords and testis-specific vasculature also developed normally (Fig. 2.2 A-D). At birth, the entire male reproductive system was intact and the testis morphology was normal in the β-catenin cKO testes (Fig. 2.2 I & J). This result was further confirmed by molecular analysis of Sertoli cell marker AMH (Fig. 2.2 E & F, n=3) and Leydig cell marker CYP17 (Fig. 2.2 G & H, n=3). These results indicated that the loss of β-catenin in the SF1-positive somatic cells did not affect the development of fetal testes.

2.4.3 Ablation of β-catenin causes the alternation of the expression of ovary-specific genes

To investigate whether the development of the ovary was affected, the whole-mount in situ hybridization for Rspo1, Wnt4 and Fst, three ovary-specific genes, was performed on fetal gonads. In the β-catenin cKO ovary, Rspo1 expression was maintained (Fig. 2.3 C, n=2), whereas the expression of Wnt4 and Fst was absence (Fig. 2.3 F & I, n=3 for Wnt4, n=2 for Fst) compared to the control (Fig. 2.3 B, E, & H). These findings indicated that β-catenin in the SF1-positive cells is required for the expressions of Wnt4 and Fst but not Rspo1.

2.4.4 Ablation of β-catenin leads to the formation of testis-specific vessel

When Rspo1, Wnt4 or Fst was inactivated, a testis-specific vasculature, coelomic vessel, appeared in the fetal ovary (52, 55, 58). In the absence of β-catenin in the fetal ovary, the ectopic formation of the coelomic vessel occurred, which was marked by staining for PECAM-1. The coelomic vessel did not form in the control ovary but was present in the control testis (Fig. 2.4 A & B, n=3). The results suggest that β-catenin is a component of the Rspo1/Wnt4/Fst pathway that inhibits testicular vasculature formation in fetal ovaries.
2.4.5 Formation of testis-specific vessel in the \( \beta \)-catenin conditional knockout ovary does not result from the activation of male pathway

The appearance of the testis-specific coelomic vessel in the \( \beta \)-catenin cKO ovaries raised the possibility that the loss of \( \beta \)-catenin allowed the activation of the testis-specific genes. Previous studies observed that \( Sox9 \) and \( Fgf9 \) were transiently expressed in the \( Wnt4 \) knockout ovaries. Hence it is proposed that \( Wnt4 \) antagonizes the expression of \( Sox9 \) and \( Fgf9 \), two factors critical for specifying the testis fate according to the observation (73). I therefore performed the whole-mount \textit{in situ} hybridization for \( Sox9 \) and \( Fgf9 \) to examine whether \( Sox9 \) and \( Fgf9 \) were upregulated and thus could be responsible for coelomic vessel formation in the \( \beta \)-catenin cKO ovaries. \( Sox9 \) and \( Fgf9 \) mRNA were expressed strongly in the control testes (Fig. 2.5 A & G, n=3 for \( Sox9 \), n=2 for \( Fgf9 \)). However, there was no evidence of upregulation of \( Sox9 \) and \( Fgf9 \) mRNA in the control or \( \beta \)-catenin cKO ovaries at 11.5 and 12.5 dpc (Fig. 2.5 B & C and H & I). In addition, immunohistochemical analysis of SOX9 protein confirmed the mRNA observation (Fig. 2.5 D-F, n=2). These data revealed that loss of \( \beta \)-catenin did not lead to activation of the testis pathway, indicating that disruption of the ovarian program and the appearance of the coelomic vessel were the direct outcomes of the loss of \( \beta \)-catenin.

2.4.6 Ablation of \( \beta \)-catenin leads to the masculinization of female embryos

By investigating the reproductive tract at birth, the development of the reproductive tract in the \( \beta \)-catenin cKO female was found to be grossly similar to that of the control female, i.e. the position of the \( \beta \)-catenin cKO ovaries was similar to the control ovaries (Fig. 2.6 A-B). However, further examination revealed that the \( \beta \)-catenin cKO female developed epididymal structure and vas deferens, both derivatives of the Wolffian duct (Fig. 2.6 D). Histological analysis using H&E staining further confirmed the epididymal structure was similar to the one in the control male (Fig. 2.6 F & G, n=2). These Wolffian duct-derived structures were absent in the control female (Fig. 2.6 C & E, n=2). The Müllerian duct derivatives such as oviduct and uterus were still intact in the \( \beta \)-catenin cKO female. Although some of the Wolffian duct derivatives were maintained, the external genitalia of the \( \beta \)-catenin cKO female was indistinguishable from that of the control.
2.4.7 Ablation of β-catenin causes the ectopic appearance of androgen-producing adrenal cells

The appearance of the epididymis-like structures and Wolfian ducts in the β-catenin cKO female embryo suggested that there was androgen production in the β-catenin cKO ovary. In the male embryos, maintenance of the Wolfian duct derivatives requires androgens produced by fetal Leydig cells. Thus, the epididymal structures and vas deferens observed in the β-catenin cKO female could result from the ectopic appearance of androgen-producing fetal Leydig cells in a manner akin to Rspo1 and Wnt4 knockout mice. To test this possibility, the immunohistochemistry for CYP17, a key enzyme in the pathway of androgen production, and Insulin-like 3(INSL3), a Leydig cell-specific factor responsible for testis descent was performed (166, 167). CYP17 expression was detected in the β-catenin cKO ovaries but was absent in the control ovaries (Fig. 2.7 A & B, n=3). However, INSL3 was not detected in either the control or β-catenin cKO ovaries (Fig. 2.7 C & D, n=3), indicating that the CYP17-positive cells were not fetal Leydig cells. In addition, in the β-catenin cKO female, the ovaries remained attached to the posterior part of the kidney and did not descend to the position adjacent to the bladder (Fig. 2.7 B), further confirming the absence of INSL3.

In addition to the fetal Leydig cells, other possible steroidogenic cells present in the fetal testis are adrenal cells (168). It has been shown that the small population of adrenal cells were only present in the testes but not in the ovaries (168, 169). Precious studies also found that adrenal-like cells positive for both Cyp17 and Cyp21 were found in the Wnt4 knockout ovaries (62). To examine whether these Cyp17-possivie cells were of adrenal origin, I performed in situ hybridization with Cyp21 probe, a steroidogenic marker of adrenal, on gonads at birth. The Cyp21-positive cells were observed exclusively in the β-catenin cKO ovary but not in the control ovary (Fig 2.7 E & F, n=4). Immunohistochemical analysis of CYP21 protein also confirms the mRNA observation. (Fig. 2.7 G & H, n=3). These data demonstrate that the pathway induced by β-catenin suppresses the appearance of the androgen-producing adrenal-like cells in fetal ovaries.

2.5 Discussion

Abolishment of β-catenin in the somatic cells of fetal gonads resulted in the ectopic formation of testis-specific vessel, appearance of androgen-producing cells and masculinization
of the female animal. These date indicate that β-catenin plays a critical role in maintaining ovarian characteristics.

β-catenin is not only a key secondary effector downstream of the canonical WNT pathway but also involved in cell-cell adhesion via cadherins, e.g., E-Cadherin, N-Cadherin, and P-Cadherin, particularly in epithelial cell types. Conditional deletion of β-catenin in the SF1-positive somatic cells could affect the ability of these cells to respond to Wnt signaling as well as their interaction with neighboring cells via cell adhesion. Previous studies have shown that E- and N-Cadherins were not present in the somatic cells of fetal gonads and that loss of P-cadherin did not affect fertility (170, 171). Furthermore, conditional deletion of β-catenin only induced phenotypes in ovaries, suggesting that loss of β-catenin did not affect general cell-cell adhesion in SF1-positive somatic cells that are the common precursors of gonads in both sexes. Although the antibody used in this study detected only membrane-bound β-catenin in the fetal gonads, this result does not exclude important nuclear functions of β-catenin. Using the same antibody, others observed nuclear β-catenin in fetal gonads only when a mutant form of β-catenin resistant to degradation was over-expressed (165). This observation suggests that under normal condition, nuclear shuttling of β-catenin is a transient and dynamic process that is difficult to monitor using immunohistochemistry. Based on the loss of membrane-bound β-catenin staining and occurrence of unique phenotypes in the β-catenin cKO gonads, I am confident that I have generated a valid functional model to study the role of β-catenin in the SF1-positive somatic cells.

**β-catenin is dispensable for the fetal testis formation**

When β-catenin was inactivated in the SF1-positive somatic cells population at 11.5 pdc, the time when the sex determination occurs, the ovary pathway, not the testis pathway, was affected. In the absence of β-catenin, molecular markers for Sertoli and Leydig cell differentiation were expressed properly and testicular structures and germ cells differentiated normally. At the time of birth, the β-catenin cKO male had virilized reproductive organs indistinguishable from the control male. Using Amh-cre line to remove β-catenin specifically in Sertoli cells starting at 15.5 dpc, others found no defects in later testis development (165). My results extend this finding by showing that β-catenin is also not required in the SF1-positive Leydig cell population during early testis morphogenesis and later maintenance. The activation of stabilized β-catenin in the somatic cell of fetal testis disrupted the testicular program and
caused male-to-female sex-reversal (172). In the present study I show that β-catenin is dispensable for normal development of fetal testis. In the Wnt4 knockout testis, an early defect in Sertoli cell differentiation and disorganized testis cords was found (173). However, these defects were not observed in my model. It is possible that WNT4 does not signal via β-catenin in the fetal testis. In the SF1/Cre conditional knockout model, β-catenin protein became undetectable starting between 11.5 dpc and 12.5 dpc. Therefore it can not be excluded the possibility that WNT4 regulates testis development via β-catenin before 11.5 dpc.

**β-catenin is a key component of the RSPO1/WNT4 pathway in the fetal ovary development**

When the function of β-catenin in the SF1-positive ovarian cells was abolished, unique changes were observed in three ovary-specific genes, Rspos1, Wnt4, and Fst. Expression of Wnt4 and Fst, but not Rspos1, was downregulated in fetal ovaries in the absence of β-catenin, placing β-catenin upstream of Wnt4 and Fst. Interestingly, in the Rspos1 knockout ovaries, Wnt4 and Fst expression was also downregulated (55). I also observed that loss of β-catenin in the SF1-positive somatic cells produced phenotypes similar to those in Rspos1 and Wnt4 knockout female. RSPO family members, including RSPO1, are known to exert their functions via β-catenin. Using the Axin2-LacZ reporter line, in which β-galactosidase activity is driven by Axin2 promoter, Chassot et al showed that the pathway downstream of β-catenin was affected in the Rspos1 knockout ovaries, suggesting a connection between RSPO1 and β-catenin in the fetal ovary (56). These data together support the model that β-catenin is an intracellular mediator of Rspos1 that stimulates Wnt4 expression in the SF1-positive somatic cells in fetal ovary. Although it is possible that β-catenin might act downstream of Rspos1, my results do not exclude the possibility that β-catenin could also be a downstream mediator of Wnt4 as found in other model systems (142, 153, 174). To further prove the possibility that β-catenin could also serve downstream of Wnt4 in the fetal ovary, I designed a rescued experiment that β-catenin was activated in the Wnt4 KO ovary. The experiments and results are described in Chapter 4.

**The ovarian pathway, induced by β-catenin, suppresses the formation of the coelomic vessel without activating the testis-determining pathway**

The appearance of the testis-specific coelomic vessel in the β-catenin cKO, as well as in Rspos1, Wnt4, and Fst knockout ovaries, indicate that these molecules are components of a
common pathway that antagonizes the formation of testis vasculature. When these ovarian genes are inactivated, the testis pathway could become active, leading to appearance of the testis characteristics. However, there was no evidence of activation of the testis pathway based on the absence of expression of the testis markers SOX9, Fgf9, and AMH in the β-catenin cKO ovaries. These observations are similar to what others have found in Rspo1, Wnt4, and Fst knockout ovaries (52, 55, 56). Transient expression of SOX9 and Fgf9 were reported in the Wnt4 knockout ovaries at 11.5 dpc, but the expression disappeared by 12.5 dpc and no other Sertoli cell markers (e.g., Amh and Desert hedgehog) were present (73). In the β-catenin cKO model, β-catenin may be inactivated too late to allow the transient expression of Sox9 and Fgf9. However, if this is the case, the appearance of the coelomic vessel, which is thought to be controlled by Sox9 and Fgf9, should not be observed in the β-catenin cKO ovaries. Therefore the present study supports the idea that some aspects of the testis-determining mechanisms, at least the formation of the coelomic vessel, may not require Sox9 and its downstream components.

**β-catenin inhibits the appearance of adrenal-like cells in the fetal ovary**

Ectopic cells that express Cyp17 were first observed in the Wnt4 knockout ovary (53). These Cyp17-positive androgen-producing cells were initially thought to be fetal Leydig cells; subsequent studies indicated that this is not the case (53, 62), as these cells expressed adrenocortical markers (175). The androgen-producing cells were also observed in the β-catenin cKO ovaries, as evident by their expression of CYP17 in the present study. The adrenal identity of these cells was further confirmed by their expression of the adrenal marker Cyp21. The lack of expression of the Leydig cell-specific INSL3 also argues that these androgen-producing cells are not fetal Leydig cells. These CYP17-positive cells in the β-catenin cKO female embryos apparently produced androgens in amounts sufficient to sustain the androgen-dependent Wolffian duct derivatives such as epididymis and vas deferens. However, the amount of androgen produced by these adrenal-like cells was not sufficient, probably due to their small number, to virilize the external genitalia. Similar phenomena were also observed in Rspo1 and Wnt4 knockout females, indicating that Rspo1, β-catenin, and Wnt4 together suppress the appearance of these adrenal-like cells in fetal ovaries (53, 55, 56). Gonadal and adrenal cells derived from a common adrenogonadal primordium are both positive for SF1. In my conditional knockout model, β-catenin was removed in both gonads and adrenals at approximately the time
of their separation. Therefore, it is possible that one function of the Rspl/β-catenin/Wnt4 pathway is to ensure proper separation of adrenal and gonadal precursors during organogenesis. It is interesting to note that the appearance of CYP17-positive adrenal-like cells and maintenance of Wolffian duct derivatives were not found in Fst knockout (52). This observation indicates that although Fst plays a role in suppressing the formation of coelomic vessel, it is not involved in inhibiting the appearance of adrenal-like cells in the fetal ovary.

It will be interesting to consider the different effects of β-catenin disruption mediated by the SF1/cre transgene in somatic cells of the ovaries, testes, and adrenal gland. All three organs are emerged from a common pool of adrenogonadal precursors that express SF1 and potentially can assume the adrenal or gonadal fates. Thereafter, the ovaries and testes arise from a bipotential gonad as directed by signals discussed above whereas the adrenocortical precursors form an adrenal primordium that is not sexually dimorphic. Despite their common origin and conserved functions as primary steroidogenic organs, the effects of β-catenin inactivation differ markedly. At one extreme, β-catenin inactivation causes severe defects in the adrenal primordium such that the organ is completely lacking in newborn mice (140). I also observed the severe phenotype of adrenal gland, in which the adrenal was extremely small at birth by inactivation of β-catenin using SF1/cre system. In contrast, the testes in newborn mice do not exhibit any structural or functional defects (Fig. 2.2), although the dysgenesis of adrenal glands precludes analysis of their postnatal function. Finally, the ovaries are affected to an intermediate degree. The ovary forms along the normal pattern, but then exhibits abnormalities that apparently reflect impairments in mechanisms that normally inhibit certain aspects of normal testis (i.e., the testes artery) or adrenal (i.e., expression of Cyp21) development.

Based on what I have observed in the β-catenin cKO mice, I proposed the following model: Rspl acts on the SF1-positive somatic cell population and then triggers intracellular signaling via β-catenin. β-catenin then stimulates the expression of Wnt4. WNT4 maintains the identify of the fetal ovary by preventing formation of the testis-specific coelomic vessel, inhibiting the appearance of ectopic androgen-producing adrenal-like cells (Fig. 2.8).

In summary, in this chapter I have provided the genetic evidence to demonstrate a sexually dimorphic role of β-catenin in the SF1-positive somatic cell population during early gonad
development. β-catenin is dispensable for testis formation and maintenance; however, it serves as a critical mediator that maintains ovarian characteristics. In addition, in the absence of β-catenin, germ cells were lost at birth, which were also found in the Rspo1, Wnt4 and Fst null mutation mice. The functional role of β-catenin in the survival of female germ cell will be described in the Chapter 3.
## 2.6 Tables and figures

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**Table 2.1 Primer sequences for genotyping**
Fig. 2.1 Immunofluorescence analysis of β-catenin, AMH, and PECAM in control and SF1/cre;Ctnnb1<sup>fl/fl</sup> testes and ovaries. (A-B) are double-staining for AMH (Sertoli cell marker in red) and β-catenin (green) in control and mutant testes at 15.5 dpc. (C-D) are the same sections in A & B stained with β-catenin (green) and DAPI (blue). (E-F) are double-staining for PECAM (germ cells and endothelial cell marker in red) and β-catenin (green) in control and mutant ovaries at 12.5 dpc. (G-H) are the same sections in E & F stained with β-catenin (green) and DAPI (blue). Arrows and asterisks indicate germ cells and somatic cells, respectively. Scale bars represent 25 and 50 µm in (A-D) and (E-H), respectively.
Fig. 2.2 Development of testis and reproductive system in control and SF1/cre;Ctnnb1<sup>fs</sup> male. (A-B) are double immunohistochemistry for the Sertoli cell marker AMH (red) and SOX9 (green) at 12.5 dpc. (C-D) are whole-mount immunohistochemistry of E13.5 testes and mesonephroi stained with PECAM antibody, a marker for vasculature and primordial germ cells. (E-F) are immunohistochemistry for Sertoli cell markers AMH and (G-H) are immunohistochemistry for Leydig cell marker CYP17 in newborn testes. (I-J) are whole-mount light field microscopic image of the urogenital system at birth. Scale bars represent 20 µm (A-B), 100 µm (C-H), and 500 µm (I-J), respectively. b= bladder; k= kidney; m= mesonephros; t= testis.
Fig. 2.3 Expression of ovarian markers in control ovaries and control testes and SF1/cre;Ctnnb1<sup>−/−</sup> ovaries. Whole-mount in situ hybridization at 12.5 dpc for Rspo<sub>1</sub> (A-C), Wnt4 (D-F), and Fst (G-H), respectively. Scale bars represent 500 µm. m= mesonephros; o= ovary; t= testis.
Fig. 2.4 Whole-mount immunohistochemistry for PECAM-1 in control and SF1/cre;Ctnnb1f⁻/⁻ ovaries and testes. (A) Control male, (B) Control female, (C) SF1/cre;Ctnnb1f⁻/⁻ female. Scale bars represent 500 µm. m= mesonephros; o= ovary; t= testis. White arrow= coelomic vessel.
Fig. 2.5 Expression of testicular markers in control ovaries and control testes and SF1/cre;Ctnnb1<sup>fl<sup>o</sup></sup> ovaries. (A-C) and (D-F) are whole-mount in situ hybridization at 11.5 dpc and 12.5 dpc for Sox9 and Fgf9 respectively. (G-I) are cross-sections of 11.5 dpc gonads stained with AMH (red) and SOX9 (green) antibodies. Scale bars represent 500 µm in A-C and G-H, 100 µm in D-F. m= mesonephros; o= ovary; t= testis.
Fig. 2.6 Development of the internal reproductive organs in control and SF1/cre;Ctnnb1fl−/− female embryos at birth. (A-B) are whole-mount light field microscopic images of the urogenital system. (C-D) are close-up images of the reproductive tracts in A & B, respectively. (E-F) are H&E stained sections that contain ovaries and associated reproductive tracts from C & D, respectively. (G) is a H&E stained section of the epididymis of a control male. Red arrows indicate the position of the ovaries. The black arrow indicates the presence of vas deferens. Scale bars represent 500 µm. b= bladder; epi= epididymis; k= kidney; o= ovary; ovi= oviduct; u= uterus.
**Fig. 2.7** Detection of markers for fetal Leydig cells and adrenal cells in control and SF1/cre;Ctnnb1<sup>−/−</sup> ovaries at birth. (A-B) are immunohistochemistry for CYP17, a steroidogenic enzyme involved in androgen production. The inset in B represents a higher magnification of the CYP17-positive cells. (C-D) are immunohistochemistry for INSL3, a Leydig cell-specific marker. (E-F) are whole-mount in situ hybridization for Cyp21, an adrenal marker. (G-H) is immunohistochemistry for CYP21. The inset in H represents a higher magnification of the CYP 21-positive cells. The red arrow indicates clusters of Cyp21-positive cells. The scale bars represent 100 µm.
Fig. 2.8 Proposed model for the involvement of β-catenin in the pathway of fetal ovary development in mice. RSPO1 acts on the SF1-positive somatic cell population and then triggers intracellular signaling via β-catenin. β-catenin then stimulates expression of Wnt4. WNT4 maintains the identity of the fetal ovary by preventing formation of the testis-specific coelomic vessel via Fst and inhibiting the appearance of ectopic androgen-producing adrenal-like cells.
Chapter 3: Ablation of β-Catenin in the Somatic Cells of Fetal Ovary Leads to the Loss of Female Gametes

3.1 Abstract

Female germ cells are essential for the organogenesis of the ovary; without them, ovarian follicles do not form and the functional and structural characteristics of ovary are lost. In this chapter, I demonstrated that a somatic cell-derived pathway in the fetal ovary was responsible for the survival of female germ cells. Inactivation of β-catenin in the steroidogenic factor 1-positive somatic cells of fetal mouse ovary resulted in germ cell loss, similar to that in the Wnt4 knockout (KO) ovary. In the absence of Wnt4 or β-catenin, female germ cells entered meiosis properly. However, they underwent apoptosis afterwards. In addition to germ cell loss, ectopic androgen production and up-regulated inhibin β B (Inhbb) expression were observed in the fetal ovary without Wnt4 or β-catenin. Therefore, I hypothesized that the loss of germ cells in the Wnt4 KO and β-catenin conditional KO (cKO) ovaries could result from ectopic androgen production and/or the upregulation of Inhbb. The persistence of germ cell loss in the β-catenin cKO embryos after treating with flutamide, an anti-androgen drug, suggested that androgens were not responsible for the loss of germ cells. Inhbb, a subunit of activins, was upregulated in the Wnt4 KO and β-catenin cKO ovaries, suggesting that Inhbb could be the cause of the loss of female germ cells. Indeed, removal of Inhbb in the Wnt4 KO ovaries prevented female germ cells from undergoing degeneration. In summary, WNT4/β-catenin signaling pathway maintains female germ cells survival by inhibiting Inhbb expression in the somatic cells. Maintenance of female germ cells hinges upon a delicate balance between positive (WNT4 and β-catenin) and negative (Inhibin β B) regulators derived from the somatic cells in the fetal ovary.
3.2 Introduction

Female germ cells not only are essential for the propagation of species but also play an important role in ovarian organogenesis. Presence of germ cells is required for the formation of follicles, the functional unit of the ovary. Disruption of germline-specific genes, such as Dazla and Factor in the germline α (Figla), leads to the degeneration of oocytes and failure in folliculogenesis (44, 176, 177). In addition, if germ cells are lost after follicle formation, characteristics of an ovary will vanish and testis-like structures will appear (178-180). Therefore, defects in female germ cell survival could have detrimental impacts on fertility and reproductive health.

Once germ cells migrate to the gonad, they start to differentiate by following their intrinsic programs as well as instructions from the somatic environment (181, 182). In mouse fetal ovary, female germ cells enter meiosis around 14.5 dpc (day post coitum) as a result of the action of mesonephros-derived retinoic acids (109, 183) and then immediately arrest at the prophase of meiosis I (98, 182). Ideas regarding the influences of somatic cells on female germ cell development have long been proposed. Gene screening schemes have yielded putative candidate genes that may play roles in this process. Among these candidates, the Wingless-type MMTV integration site (Wnt) family of genes, including Wnt4, Wnt5a, Wnt6, and Wnt9a, are found to be expressed in the somatic cells of fetal ovary (54, 155). WNT proteins are known for their roles in cell fate decision and cell cycle regulation (127). These ovarian WNTs may work synergistically or redundantly to regulate female germ cell development. To my knowledge, WNT proteins elicit their functions through either the canonical or non-canonical WNT pathway depending on the tissue. In my research, I focused on the canonical WNT pathway in the development of fetal ovary. As I mentioned in the Chapter 2, β-catenin is the key mediator of the canonical WNT pathway. Thus, to learn the influences of WNT/β-catenin pathway on female germ cell development, I inactivated β-catenin specifically in the SF-1 positive somatic cells. In Chapter 2, I demonstrated that β-catenin participated in the WNT4/RSPO1 pathway to maintain ovarian identity. In this chapter, I further demonstrated that in the absence of β-catenin in the somatic cells of fetal ovary, germ cells underwent massive demise as they did in the Wnt4 knockout mice. In addition, I investigated what caused germ cell death in the absence of WNT4/β-catenin signaling from the somatic cells of fetal ovary. To do this, I injected flutamide, an androgen
antagonist, into the pregnant mice carrying β-catenin KO embryos. I found germ cell loss still occurred in the KO ovaries without the involvement of androgens in this process. Germ cell number was restored in the Wnt4 and Inhbb double KO ovary indicated that the upregulation of Inhbb was responsible for the loss of germ cells in the Wnt4 KO ovary. These results demonstrated that WNT4 and β-catenin suppressed the expression of activin βB. When the WNT4/β-catenin pathway is inactivated, upregulation of activin βB leads to the loss of female germ cells. Thus, to learn the influences of WNT/β-catenin pathway on female germ cell development, I inactivated β-catenin specifically in the SF-1 positive somatic cells. In the Chapter 2, I demonstrated that β-catenin participated in the WNT4/RSPPO1 pathway to maintain the ovarian identity. In this chapter, I further demonstrated that in the absence of β-catenin in the somatic cells of fetal ovary, germ cells underwent massive demise as they did in the WNT4 knockout mice. In addition, I investigated what caused germ cell death in the absence of WNT4/β-catenin signaling from the somatic cells of fetal ovary. To do this, I injected flutamide, an androgen antagonist, into the pregnant mice carrying β-catenin KO embryos. I found germ cell loss still occurred in the KO ovaries without the involvement of androgens in this process. Germ cell number was restored in the Wnt4 and Inhbb double KO ovary indicating that the upregulation of Inhbb was responsible for the loss of germ cells in the Wnt4 KO ovary. These results demonstrated that WNT4 and β-catenin suppressed the expression of Inhbb. When the WNT4/β-catenin pathway was inactivated, upregulation of Inhbb led to the loss of female germ cells.

3.3 Experimental Procedures

3.3.1 Animals

Transgenic mice (SF-1/cre) carrying Cre recombinase under the control of the Steroidogenic factor 1 (SF-1) regulatory element (159) and floxed β-catenin mice (Ctnnb1floxed/floxed or B6.129-Ctnnb1tm2Kem/KnwJ, Jax#004152) (160) were maintained on a B6 background. To increase the efficiency of production of homozygous null alleles and decrease the incidence of mosaic deletion, SF-1/cre;Ctnnb1−/− embryos were be generated. The detail breeding schemes were list in Chapter 2 in 2.3.1. Wnt4+/− mice were obtained from Jackson Laboratory (strain 129-Wnt4tm/Amc). Female and male mice were housed together and checked for the
presence of a vaginal plug the next morning. To obtain the \( Wnt4 \) and activin \( \beta B \) double knockout mice (\( Wnt4^{+/+}; Inhbb^{-/-} \)), \( Wnt4^{+/+} \) mice were mated to \( Inhbb^{+/+} \) mice to generate \( Wnt4^{+/+}; Inhbb^{+/+} \) double heterozygotes. \( Wnt4^{+/+}; Inhbb^{+/+} \) double heterozygotes were mated to generate \( Wnt4^{-/-}; Inhbb^{-/-} \) double knockout mice. The day when the vaginal plug was detected in the mated female was considered as 0.5 days post coitum (0.5 dpc).

### 3.3.2 Genotyping

For genotyping, tail clippings were digested in 200 µl of 50 mM sodium hydroxide for 30-45 minutes at 95°C, then 100 µl of 1 M Tris HCl, pH=8 were added and mixed before centrifugation at 10,000 g for 5 minutes at room temperature. Samples were then subjected to standard PCR using gene-specific primers. A 25 µl standard PCR reaction was set up as follows: 1 µl of tail DNA, 12.5 µl of 2X PCR Mango Mix (Bioline), 1.25 µl of 10 mM gene-specific primers and 10.25 µl of double distilled water. All PCR reactions were run using the BIO-RAD MyCycler Thermal Cycler followed by running on 1.5 % or 2% TAE gel at 100 volts. The primers and PCR reactions for genotyping were listed in Table 3.1 and Appendix A.

### 3.3.3 Histology

Tissues were fixed in 4% paraformaldehyde in PBS overnight at 4°C, processed through graded ethanol series and Histo-clean (International Diagnostics), and eventually embedded in paraffin. Five µm sections were obtained and rehydrated for hematoxylin and eosin staining (2 minutes for hematoxylin and 30 seconds for eosin). Slides were mounted using Permount.

### 3.3.4 Immunohistochemistry

Samples were fixed in 4% paraformaldehyde overnight at 4 °C and then washed in PBS for 5 minutes (3 times). Samples were put through a sucrose gradient (10%, 15% and 20 %) and incubated in 1:1 20% sucrose and OCT freezing media (Tissue-Tek) overnight at 4 °C. Samples were embedded in 1:3 20% sucrose and OCT mix and cut to 10 µm thick frozen sections. Sections were washed with PBS and then blocked in the blocking solution (5% heat-inactivated donkey serum and 0.1% Triton X-100 in PBS) for 1 hour at room temperature. Primary antibodies were added to the blocking solution and incubated with sections at 4 °C overnight. Sections were then washed with the washing solution (1% heat inactivated donkey serum and
0.1% Triton X-100 in PBS) followed by incubation in the blocking solution with the corresponding secondary anybodies. Sections were then washed with the washing solution and mounted with DAPI antifade reagent. The sources and dilution of primary antibodies were: rat monoclonal antibody against germ cell nuclear fraction (TRA98, 1:000, a gift from H. Tanaka), the rabbit polyclonal antibody against cleaved caspases-3 (1:200; Cell Signaling), and rabbit polyclonal antibody against CYP17 (1:100, a gift from B. Hales), a rabbit polyclonal antibody against phosphorylated histone-2AX (1:100, Upstate). All the secondary antibodies were purchased from Jackson Immunochemical and a 1:200 dilution was used.

3.3.5 Chromosome smear and immunostaining

Fetal germ cell chromosome smear and immunostaining were performed according to the protocol described in (184). Briefly, ovaries from 15.5 dpc embryos were put in a 24-well dish with hypoextraction buffer (15 mM Tris,pH 8.2, 50 mM sucrose, 20 mM citrate, 5mM EDTA, pH 8.2, 0.5 mM DTT, 0.09 mg/ml PMSF, collagenase, 0.5mg/ml ) at least 30 minutes. Then one ovary was placed in a 10 ml drop of 0.1 M sucrose on the slide and another 10ml drop of sucrose was added. The ovaries were dispersed by repetitive pipetting. Ovary suspension was then placed onto the slide coated with fixative (0.1% paraformaldehyde, pH 9.2, 0.1% Triton 100). The samples were placed in a humid chamber for 4h then slides were gently washed three times (5 minutes each) in 1:250 photo-flo (Kodak) in water. Slides were air-dried and stored in -20 °C up to 1 month.

For immunostaining of spread chromatin, slides were washed three times (10 minutes each) in 10% antibody dilution buffer (ADB: 10% donkey serum, 3% BSA, and 0.05% Triton-X in phosphate-buffered saline or PBS). Then slides were incubated with anti-SCP3 antibody (1:500, Abcam) in a humid chamber overnight at 4°C. Next day, samples were washed three times (10 minutes each) in 10% antibody dilution buffer. Slides were then incubated with secondary antibody in the dark for 2hs at room temperature then washed three times (5 minutes each) in PBS. Slides were air-dried and mounted with DAPI.

3.3.6 Flutamide treatments

Flutamide (F9397, Sigma-Aldrich) was dissolved in 1:1 (vol/vol) mixture of absolute ethanol and sesame oil. Ten pregnant Ctnnb1floxed/floxed female mice that were plugged by SF-1/cre;
Ctnnb1+/− male were injected daily with flutamide subcutaneously (100 mg/kg/daily) from 12.5 dpc until birth (175). Five pregnant mice from the same breeding scheme were treated with the vehicle (sesame oil) from 12.5 dpc until birth (control group).

3.3.7 Mini preparation of Inhbb plasmid

Each single colony that carries Inhbb plasmid was picked from the Luria-broth ampicillin agar plate and dropped into a separate tube of 3mL LB with ampicillin and grow at 37°C while shaking for 16 hours. The Qiagen mini-prep kit was used for DNA preparation (Cat. no. 27106, Qiagen, USA). In brief, the bacteria solution was collected in 1.5 ml tubes. The tubes were centrifuged at maximum speed for 1 minute and the supernatant was removed. Then, 250 μl of cold buffer P1 were added to the solution and the pellet was suspended using vortexing. Next, 250 μl of buffer P2 were added in the tube and mixed with buffer P1 thoroughly by inverting the tube 4–6 times incubated at room temperature. Then 350 μl of buffer N3 were added and mixed immediately and thoroughly by inverting the tube 4–6 times. Samples then were centrifuged at 10,000 g for 10 minutes at room temperature and the supernatant was transferred to the QIAprep spin column followed by washing with buffer PB and buffer PE. Finally, DNA was eluted from the column by adding TE buffer.

3.3.8 Inhbb RNA probe synthesis

Five μg of Inhbb plasmid were treated with the appropriate restriction enzyme for linearization then purified by mixing with 1 volume phenol/ chloroform /Isoamyl alcohol (25:24:1) and centrifuging at 10,000 g for 10 minutes. Supernatant was moved to a new tube and the residual phenol was removed by one volume of chloroform/isoamyl alcohol (24:1). DNA was precipitated by adding 2 volumes of 100% Ethanol and incubated at -20 °C for at least 2 hours. DNA was then recovered by centrifugation at 10,000 g, 4 °C for 30 minutes and washed with ice-cold 70 % ethanol. The in vitro transcription reaction contained: 1 μg of linear plasmid, 2 μl of 10X transcription reaction buffer, 2 μl of Digoxigenin nucleotide mix, 1 μl of appropriate RNA Polymerase, and DEPC-water to a volume of 20 μL. Samples were incubated at 37°C for 2 hours. Then 0.8 μl of 0.5M EDTA were added to stop the reaction. Next, 1 μl of 10M Lithium chloride and 75 μl cold 100% ethanol were added to the reaction mixture. Samples were placed at -20°C for at least 2 hours followed by centrifugation at 10,000 g speed for 30 minutes at 4°C.
After the centrifugation, the supernatant was removed and the pellet was washed with ice cold 75% DEPC-ethanol and re-suspended in 20 µl DEPC water. Finally, the probe was diluted 1:1 with hybridization buffer (5x SSC pH 5.0, 50% formamide, 0.1% CHAPS, 0.1% Tween20, 1mg/ml yeast tRNA, 50 µg/ml Heparin, and 5mM EDTA pH 8.0) and stored at -20 °C. A gel check was also utilized to determine if the probe was transcribed correctly.

3.3.9 Whole mount in situ hybridization

Tissues were fixed overnight in 4% paraformaldehyde in PBS at 4°C and dehydrated through a methanol gradient (25%, 50%, 70%, and 100%) in PTW (0.1% Tween20 in DEPC-PBS). Samples were stored in 100% methanol at -20°C. In situ hybridization was processed according to the standard non-radioisotopic procedure using digoxigenin-labeled RNA probes. In brief, samples were dehydrated through a methanol gradient then treated with proteinase K (10 mg/ml) at 37°C for 12 minutes followed by fixation in 4% paraformaldehyde/0.1% glutaldehyde immediately at room temperature for 20 minutes. Samples were pre-hybridized in hybridization buffer (5x SSC pH 5.0, 50% formamide, 0.1% CHAPS, 0.1% Tween20, 1mg/ml Yeast tRNA, 50 µg/ml Heparin, and 5mM EDTA pH 8.0) at 65 °C for 1 hour. Then digoxigenin-labeled RNA probe was added into the solution and samples were rotated in an oven at 65°C overnight (12-16 hours). Samples were washed the next day in pre-warmed hybridization buffer followed by MABTL (5% MAB, 0.1% Tween20 and 0.05% Levamisol). Samples were incubated in 20% sheep serum in MABTL blocking solution at room temperature for 2 hours followed by incubation in alkaline phosphatase-conjugated anti-digoxigenin at 4°C on shaker overnight. On the third day, after washing in MABTL three times for 1 hour each, samples were incubated in alkaline phosphates substrate in NTMTL (0.1 M NaCl, 0.01 M Tris-HCl pH 9.5, 0.05 M MgCl2, 1% Tween20, 0.05% Levamisol) for color development.

3.3.10 Gonad culture

Fetal ovaries with mesonephros attached were dissected at 12.5 dpc and were cultured on a 1.5% agar block in Dulbecco’s minimal Eagle medium (DMEM, supplemented with 10% fetal calf serum and 50 µg/ml of ampicillin) with 5% CO2. Affi-Gel Blue Gel beads (Bio-Rad Cat. No. 153-7302) was soaked in human activin B (200 ng/ml in PBS, R&D) or in PBS (as control) at 37°C for 1 hour. The soaked beads then were added to the top of the ovaries and cultured for
96 hours. The medium was changed every 24 hours. The cultured ovaries were collected and fixed in 4% paraformaldehyde at room temperature for 1 hour followed by washed with PBS.

3.4 Results

3.4.1 The number of germ cells was dramatically decreased in the absence of β-catenin in the somatic cells of fetal ovary

Differentiation and survival of female germ cells require a unique somatic environment in the fetal ovary. The disturbance of the ovarian pathway and/or other phenotypes in the β-catenin conditional knockout (SF1/cre;Ctnnb1fl/fl; or β-catenin cKO) ovaries that I showed in the Chapter 2 could affect germ cell development. To learn what effects in the absence of β-catenin in the somatic cell of fetal ovary, I performed immunohistochemistry for TRA98, a germ cell marker, (162) on various stages of control and the β-catenin cKO ovaries. I found that the number of germ cells was similar between the control and the β-catenin cKO ovaries at 15.5 dpc (Fig. 3.1A & B, n=3). A significant decrease in germ cell numbers became apparent at 16.5 dpc (Fig. 3.1C & D, n=3) and female germ cells in the β-catenin cKO ovaries were almost completely lost at birth (Fig. 3.1E & F, n=5). H&E staining further revealed the absence of female germ cells in the β-catenin cKO ovaries (Fig. 3.1G & H), indicating that the absence of TRA98 staining was due to germ cell loss instead of decreased TRA98 expression.

3.4.2 Ablation of β-catenin in the somatic cells of fetal ovary causes the program cell death of female germ cells

To assess whether the loss of germ cells resulted from the apoptosis in the β-catenin cKO ovaries, a time course analysis of female germ cells using the double immunohistochemistry with the apoptotic marker cleaved caspase 3 and germ cell marker TRA98 were performed on control and β-catenin cKO ovaries. Numbers of TRA98-positive germ cells were similar between control and β-catenin cKO ovaries at 15.5 dpc (Fig. 3.2 A & B). However, female germ cell numbers steadily declined in the β-catenin cKO ovaries at 17.5 dpc (Fig. 3.2 C & D, n=3) and at birth, only a few germ cells remained in the β-catenin cKO ovaries (Fig. 3.2 E & F, n=3). Starting at 17.5 dpc, numbers of female germ cells that underwent apoptosis increased significantly in the β-catenin cKO ovaries (double positive for cleaved-caspase 3 and TRA98,
magnified image in Fig. 1D). On average, only one or two germ cells underwent apoptosis per section in control ovaries (Fig. 3.2 A) but more than 5 apoptotic germ cells per section were observed in the β-catenin cKO ovaries (Fig. 3.2 B), indicating that the function of β-catenin in the SF1-positive somatic cells is to prevent female germ cells from undergoing apoptosis.

3.4.3 Ablation of β-catenin in the somatic cells of fetal ovary does not affect the initiation of meiosis of female germ cells

Defects in the meiotic machinery are a major cause for loss of female germ cells (185). To assess whether the germ cells enter meiosis properly, I examined the expression of phosphorylated gamma histone 2AX (rH2AX), a meiotic marker. I found similar expression patterns between β-catenin cKO and control ovaries (Fig. 3.3 A & B). To further evaluate whether progression of meiosis was compromised in the absence of β-catenin, I performed immunostaining for synaptonemal complex protein 3 (SCP3) on a chromosome smear obtained from female germ cells at 15.5 dpc and 16.5 dpc. Staining of SCP3, a scaffold protein formed on prophase I of meiosis, allowed the progression of meiosis in female germ cells to be monitored. In the absence of β-catenin, most female germ cells entered and progressed through prophase I of meiosis (zygotene and pachytene stages according to (96) indistinguishable from the control (Fig. 3.3 C-F). These results together indicate that the β-catenin is not required for the initiation of meiosis but is essential for the survival of meiotic germ cells.

3.4.4 Ectopic production of androgens is not responsible for germ cell loss in the β-catenin conditional knockout ovary

In addition to germ cell loss, inactivation of Wnt4 or β-catenin resulted in ectopic appearance of androgen-producing CYP17-positive cells in the ovary as I showed in the Chapter 2 and (62). These ectopic CYP17-positive cells produce sufficient androgen to maintain androgen-dependent male reproductive organs such as epididymis and vas deferens in the Wnt4 (53) and β-catenin cKO female. To examine whether ectopic androgen production is responsible for the loss of germ cells, an anti-androgen drug, flutamide, were daily injected from 12.5 dpc to birth into pregnant female mice carrying β-catenin cKO embryos. Flutamide is a potent androgen antagonist that has been widely used to block androgenic effects for clinical treatment of prostate
cancer and for basic research on androgen action during embryogenesis (186). Flutamide injection efficiently blocked the masculinizing effects of androgens in the β-catenin cKO female embryos based on the fact that male reproductive characteristics such as the epididymis were inhibited (Fig. 3.4 D & H) compared to the cKO female without flutamide treatment (Fig. 3.4 B & F). To further confirm that androgen functions were properly inhibited, I examined control male embryos exposed to flutamide in utero. I observed different levels of underdeveloped testis and other male reproductive organs compared to the vehicle-treated control (Fig. 3.5). These results were similar to what was reported in the literature (175), indicating that the flutamide treatment was sufficient to block androgen action in my system. However, regardless the presence or absence of flutamide treatment, loss of female germ cells was still observed in the β-catenin cKO ovaries at birth (Fig. 3.4 J & L). Flutamide treatment had no effects on development of the female reproductive systems and female germ cells in the control females (Fig. 3.4 A, C, E, G, I, & K). These results demonstrate that loss of germ cells in the β-catenin cKO ovary does not result from ectopic androgen production.

3.4.5 Genetic identification of inhibin β B as the factor downstream of WNT4/β-catenin that is responsible for inducing female germ cell loss

The TGFβ superfamily has been shown to play a role in inducing apoptosis (187, 188). In the case of freemartins, where female embryos were exposed to anti-Müllerian hormone, a member of the TGFβ superfamily, germ cell loss was observed (189, 190). Previous studies from our lab found that the mRNA expression of inhibin βB (Inhbb) was significantly elevated in the Wnt4 KO ovaries (191). In this study, I also observed the mRNA expression of Inhbb was upregulated in the β-catenin cKO ovary compared to the controls (Fig. 4 A-C, n=3) as in the Wnt4 KO ovary, indicating WNT4 and β-catenin were in the same pathway to suppress the expression of Inhbb in the fetal ovary. I therefore hypothesized that if elevated Inhbb is indeed responsible for female germ cell loss in the absence of Wnt4, removal of Inhbb in the Wnt4 KO background should reverse this phenotype. Indeed, in the Wnt4<sup>−/−</sup>; Inhbb<sup>−/−</sup> double KO ovary, female germ cell number was significantly increased compared to the Wnt4 single KO at birth (Fig. 3.6 D-F, n=3). Female germ cells in the Wnt4<sup>−/−</sup>; Inhbb<sup>−/−</sup> double KO ovary entered meiosis properly as evident by double immunostaining for SCP3 and TRA98 (Fig. 3.6 J-L, n=3 for Wnt4<sup>−/−</sup>; Inhbb<sup>−/−</sup> and control, n=2 for Wnt4 signal KO). Similar to the Wnt4 single knockout ovary,
ectopic CYP17-positive cells and epididymal structure were found in the Wnt4−/−; Inhbb−/− double KO ovary but were absence in the control female at birth (Fig. 3.6 D-I, n=3 for CYP17 staining, n=4 for epididymal structures), further supporting that ectopic production of androgen is not responsible for the loss of female germ cells.

3.4.6 Gonad culture with exogenous activin B does not cause germ cell deaths

To further investigate whether activin B caused the death of germ cells, I applied an in vitro gain-of-function experiment by culturing fetal ovaries with the presence or absence of the exogenous human activin B. Using the whole-mount immunostaining with PECAM-1 antibody, which marked vasculature and primordial germ cells in gonad, I observed the formation of coelomic vessel in the fetal ovary culture with the exogenous activin B (Fig. 3.7 A, n=2), indicating that the exogenous activin B treatment was functional. However, the visual inspection showed that the intensity of the expression of TRA98-positive cells was similar between control and activin B treatment groups (Fig. 3.7 C & D, n=2), suggesting that the exogenous activin B did not affect the survival of female germ cells. In addition, germ cells were positive for γHirtone2Ax in both groups indicating they underwent meiosis. The observation of meiosis suggested that the culture condition was sufficient for maintaining germ cell survival. These results of in vitro gonad culture indicated that the treatment of exogenous activin B did not cause the death of female germ cells.

3.5 Discussion

β-catenin in the SF1-positive somatic cells is essential for female germ cell survival but not required for the entry of meiosis

Somatic cells of the fetal ovary are the supporting cells that nurture the germ cells and provide them the proper environment to grow. In vitro experiments using human and mouse ovarian tissues demonstrated that factors such as Kit ligand, leukemia inhibiting factor, bone morphogenetic factor 4, basic fibroblast growth factor, and activin A stimulate folliculogenesis and differentiation and survival of germ cells in culture (192, 193). However, in vivo evidence is lacking to support a functional role of these factors.

In this chapter, I demonstrated that the removal of β-catenin from the SF1-positive somatic cells of fetal ovaries caused loss of female germ cells. Similar female germ cell demise was also
found in the Rspo1, Wnt4, and Fst knockout ovaries (52, 53, 55, 56). The loss of female germ cells could be due to improper differentiation of somatic cells in the absence of β-catenin, appearance of the coelomic vessel, ectopic production of androgens and/or adrenal hormones from the adrenal-like cells, or a combination of these effects. Based on the time course analysis of chromosome smears of germ cells and examination of expression of SCP3 and γH2AX, I observed that female germ cells entered meiosis and progressed to meiosis prophase I normally in the β-catenin cKO ovary. In addition, the female germ cells also enter meiosis normally in Wnt4 and Fst knockout ovaries (52). The initiation of meiosis is influenced by the retinoic acid (RA) signaling from mesonephros (109, 110). Without the present of RA in the fetal ovary, the expression of Stra8 (stimulated by retinoic acid gene 8) is absent and female germ cell do not initiate the meiosis (110, 111). Thus, my findings suggest that the retinoic acid pathway that regulates meiosis entry is probably not affected by the absence of Wnt4/β-catenin. It is interesting to notice that in the β-catenin cKO testis, male germ cells remained undisturbed, further supporting that β-catenin is only required for development of the ovary (Chapter 2, Fig 2.2).

Studies on the R-spondin1 (Rspo1) KO mice showed that Rspo1 was the upstream regulator of Wnt4 and β-catenin in ovarian development (55, 56). In the absence of Rspo1, components of the RA pathway were not significantly affected (56). Although a decrease in germ cell numbers was reported in the Rspo1 KO ovary at 14.5 and 16.5 dpc, germ cell entry into meiosis appeared to be normal based on the SCP3 staining (55). The possibility of a direct action of RSPO1 on female germ cells remains to be determined.

**Wnt4/β-catenin signaling pathway suppresses the expression of inhibin βb to maintain the survival of female germ cells**

Germ cell loss still occurred in β-catenin cKO fetal ovaries after flutamide treatment, therefore excluding the involvement of androgens. Furthermore, androgen receptors are not present in germ cells at fetal stages (194), supporting the conclusion that ectopic androgen was not responsible for the death of germ cell in Wnt4 KO and β-catenin cKO fetal ovaries (Fig. 3.9). Rescue of the germ cell loss phenotype in the Wnt4−/−; Inhbb−/− double KO ovary provides a genetic link implicating Inhbb as the molecule responsible for germ cell demise. Inhbb encodes the subunit for inhibin B and activin B. Germ cells are known to express receptors (Acrr-IB and
ActR-IIB) for activins (195). The ectopic production of activin B from somatic cells of the fetal ovary in the absence of \textit{Wnt4} and β-catenin could therefore act directly on female germ cells and cause their death. Using Transcription Element Search System (TESS) program, I found several putative LEF/TCF response elements in the promoter region of \textit{Inhbb}. β-catenin could bind to these response elements and suppress the expression of \textit{Inhbb} in the fetal ovary (Fig. 3.8). It will be interesting to further study whether or not β-catenin directly regulate \textit{Inhbb} in the development of fetal ovary.

**FST participates in the WNT4/β-catenin pathway to antagonize Activin B, the protein product of inhibin βb in the fetal ovary**

The germ cell loss phenotype was also observed in the follistatin (\textit{Fst}) knockout fetal ovaries (52). Based on a genetic epistasis experiment, \textit{Fst} acts downstream of WNT4 (52). Furthermore, expression of mouse \textit{Fst} is dependent upon a consensus LEF/TCF binding site in the promoter region (141, 142), and expression of \textit{Fst} was lost in the β-catenin cKO ovary as I showed in the Chapter 2, placing \textit{Fst} downstream of β-catenin. FST is known to bind activins with high affinity, therefore preventing activins from activating their receptors (196). Expression of \textit{Inhbb} mRNA is present in mouse gonads of both sexes at 11.5 dpc. Its expression was down-regulated but remained detectable in the ovary at 12.5 dpc (191). Interestingly, in contrast to the \textit{Wnt4} and β-catenin cKO ovary where \textit{Inhbb} expression was upregulated, \textit{Inhbb mRNA} expression levels were not altered in the absence of \textit{Fst} (191). Thus, the function of FST could be to antagonize and inhibit the action of the residual activin B to prevent it from affecting female germ cell survival. It is possible that Wnt4/β-catenin acts at two levels to block the effects of activin B on germ cells, by downregulating transcription of \textit{Inhbb} and by activating FST to antagonize activin B protein.

**Maintenance of female germ cells hinges upon a delicate balance between the positive and negative regulators derived from the somatic cells in the fetal ovary**

It is known that oocytes start entering apoptosis prenatally, therefore leaving a finite number of oocytes for the rest of the reproductive life of female individuals. At least two hypotheses have been proposed for the cause of female germ cell demise during embryogenesis
The first possible mechanism is that abnormal oocytes with defects on theirs chromosomes or mitochondrial genomes are eliminated from the oocyte pool via intrinsic check-point mechanism (125). For example, the patients with Turner syndrome (XO) who have disorder of sex chromosomes, are often associated with gonadal dysgenesis with absence or near absence of germ cells in the gonads (125). Another possibility is that the somatic cell environment controls the numbers of female germ cells. For example, in the absence of Kit ligand from the somatic cells, germ cells undergo apoptosis and are eventually degenerated (89). Although I did not observe that the death of germ cell occur in the gonad culturing with exogenous Activin B, these preliminary results of in vitro gonad culture did not eliminate the possibility that activin B was the cause of the loss of female germ cells in the absence of WNT4/β-catenin signaling in the fetal ovary. I propose two possible explanations for getting those outcomes from this in vitro gonad culture. First, the concentration of the exogenous activin B applied in this experiment might not be sufficient to affect female germ cell survival, assuming the exogenous activin B indeed has effects. Further experiments with different concentrations of the exogenous activin B are needed to gauge the threshold level. Second, follistatin, an Activin-binding protein, was highly expressed in the fetal ovary and might suppress the function of Activin B. Nevertheless, using genetic approach, I have demonstrated that the upregulation of Inhbb resulted in the death of female germ cells in the absence of Wnt4/β-catenin signaling from the somatic cells.

The balance between somatic cell signaling (WNT4/β-catenin) and activin B (the protein product of Inhbb) is critical for the maintenance of female germ cells during embryonic stage (Fig. 3.9). It is possible that increasing germ cell apoptosis close to birth is the result of a shifted balance toward action of activin B. If my hypothesis is correct, one would predict that loss of Inhbb should lead to decrease in germ cell apoptosis and presumably more oocytes in the ovary. Inhbb knockout females were fertile despite an increase in length of gestation and a decrease in ability of nursing (197). It remains to be determined whether more oocytes are present in the Inhbb knockout ovary.

Involvement of WNT/β-catenin pathway in regulating proliferation of primordial germ cell (PGCs) is evident in Drosophila and mouse. Activation of β-catenin in PGCs promotes proliferation in Drosophila whereas it delays cell cycle progression in mouse (198, 199). In this chapter, I showed an essential role of β-catenin in somatic cells in controlling female germ cell proliferation.
numbers. Wnt4/β-catenin, in the somatic cells, acts as a suppressor of activin βB, a negative regulator of female germ cell survival. In summary, these data together provide genetic evidence that is essential for maintenance of female germ cell survival.
### 3.6 Tables and figures

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<td>2. TGGGATGGAATGGTGGTGCAG</td>
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**Table 3.1 Primer sequences for genotyping**
Fig. 3.1 Development of female germ cells in the control and SF1/cre;Ctnnb1β (β-catenin cKO) ovaries. (A-F) are immunohistochemistry for germ cell nuclear marker TRA98 on E15.5 (A-B), E16.5 (C-D) and birth (E-F). (G-H) are H&E stained ovary sections at the time of birth. Black arrows indicate germ cells with large and round nuclei. Only one abnormal-looking germ cell is found in (H). The scale bars are 100 μm in (A-F) and 50 μm in (G-H).
Fig. 3.2 Time course analysis of germ cell development by immunohistochemistry for germ cell marker TRA98 (red) and apoptotic marker cleaved caspase 3 (green). (A, C, E) control SF1/cre;Ctnnb1\(^{f/+}\) ovaries. (B, D, F) β-catenin conditional KO SF1/cre;Ctnnb1\(^{f/-}\) ovaries. The arrows indicate cells that are double-positive (yellow) for TRA98 and cleaved caspases 3. Scale bar represent 100 µm.
**Fig. 3.3 Effects of somatic cell ablation of β-catenin on germ cell entry into meiosis.** (A-B) are immunohistochemistry for meiosis marker γHiston2AX (green). Pictures in the insets are higher magnification of individual germ cells from the sections. (C-F) Analysis of the meiotic status of germ cells via immunohistochemistry for SCP3 on germ cell smear obtained from the control and β-catenin conditional KO ovaries at 15.5 dpc. The samples were counterstained with nuclear DAPI (blue). Scale bar represent 100 µm in A-B and 10 µm in C-F.
**Fig. 3.4 Effects of flutamide treatment on development of reproductive tract and germ cells in β-catenin conditional KO female.** (A-H) Whole mount images of reproductive tracts and ovaries and (I-L) immunohistochemistry for TRA98 were performed on the ovary of control female without flutamide treatment (A, E, I), β-catenin conditional KO female without flutamide treatment (B, F, J), β-catenin conditional KO with flutamide treatment (D, H, L), and control female with flutamide treatment (C, G, K). O= Ovary. Arrow= epididymis, arrowhead= Ovid duct. Scale bar represent 500 µm in A-F and 100 µm in G-I.
Fig. 3.5 Effects of flutamide treatment on development of reproductive tract and testis in male mice. (A-C) Whole mount images of reproductive tracts and testis. (D) whole mount images of testes. The testis without flutamide treatment is on the left and with flutamide treatment is on the right. (E) H&E stained testis sections at the time of birth. T= testis. Epi= epididymis, Scale bar represent 500 µm.
Fig. 3.6 Involvement of Inhbb in germ cell loss in the absence of Wnt4. (A-C) Whole mount in situ hybridization for Inhbb was performed on control testis (SF1/cre;Ctnnb<sup>f/+</sup> or Ctnnb<sup>1f/c</sup>) (A), control ovary (SF1/cre;Ctnnb<sup>f/+</sup> or Ctnnb<sup>1f/c</sup>) (B), and β-catenin cKO ovary (SF1/cre;Ctnnb<sup>f/c</sup>) (C) at 13.5 dpc. (n=3 for each genotypes). o= ovary, m= mesonephros. (D-F) Immunohistochemistry for TRA98 (red) and CYP17 (green) was performed on ovary sections from control (Wnt4<sup>+/−</sup>; Inhbb<sup>+/−</sup>), Wnt4 single KO and Wnt4; Inhbb double KO ovary at birth. (G) Light field microscopic images of the reproductive tract were taken from control female, Wnt4 single KO, and Wnt4; Inhbb double KO females at birth. Arrowheads indicate oviduct and arrows indicate epididymal structure. o=ovary. (J-L) Immunohistochemistry for TRA98 (red) and SCP3 (green) were performed on ovary sections from control, Wnt4 single KO and Wnt4; Inhbb double KO ovary at 15.5 dpc. The insets are the images of a higher magnification of cells double-positive (yellow) for TRA98 and SCP3. Scale bar represent 250 µm in A-C, G-I and 100 µm in D-F, J-L.
**Fig. 3.7 The effects of exogenous activin B on female germ cell development.** (A) Whole mount PECAM-1 image on fetal ovary cultured with Activin B. White arrow indicates the coelomic vessel. (B-C) Immunostaining with a germ cell marker, TRA98, and a meiosis marker, γHston2Ax, on fetal ovary cultured with PBS (A) and fetal ovary cultured with Activin B (B). Insets are higher magnification of individual germ cells from the sections. Scale bar represent 500 µm in A, 100 µm in C-D.
Fig. 3. The putative LEF/TCF response elements in the promoter region of Inhbb. Bold and highlight sequence denotes scores of a perfect match to a LEF/TCF consensus string sequence was higher than 10. Highlight sequence denotes the scores were between 10-8. ATG indicates the translation start sties.
Fig. 3.9 A proposed model for the somatic cell-derived pathway on female germ cell survival. In the mouse fetal ovary, WNT4/β-catenin signaling repress the expression of activin βB or Inhbb, which causes loss of meiotic germ cell. WNT4/β-catenin also stimulates the production of follistatin (Fst), which acts to antagonize the activity of Inhbb. The WNT4/β-catenin pathway also prevents the ectopic production of androgens in the fetal ovary, which is not responsible for the germ cell loss.
Chapter 4: Activation of β-Catenin in the Somatic Cells of Wnt4 Knockout Ovary Prevents the Wnt4 Knockout Phenotypes from Appearing in Female

4.1 Abstract

WNT4 and β-catenin both are required for the development of fetal ovary. Abolishment of β-catenin in the SF1-positive somatic cells caused the decreased expression of Wnt4 mRNA, indicating WNT4 is the downstream of β-catenin. However, WNT4, a secreted protein, might also signal via β-catenin to regulate its own expression in the fetal ovary. To test this hypothesis, I generated a genetic model in which β-catenin was ectopically activated in the SF1-positive somatic cells of the Wnt4 KO fetal ovary. The ectopic appearance of androgen producing cells and masculinized female embryos were no longer existent in the Wnt4 KO ovary with activated β-catenin. In addition, the number of germ cells was restored to levels similar to the normal ovary. These data indicate that WNT4 elicits its function through β-catenin in the fetal ovary. It is possible that WNT4 reregulates its own expression via β-catenin. Chromatin immnuoprecipitation (ChIP) assay revealed that β-catenin did not regulate the expression of Fst via binding its proximal promoter region. A RT-PCR experiment showed that Frizzled (Fzd) 1, Fzd 4, Fzd 5 and Fzd 7, the receptors of WNT signaling pathway, were present in the fetal ovary. These Fzds could be the potential FZD receptors for WNT4 in the embryonic ovary. In summary, activation of the stabilized form of β-catenin in the Wnt4 KO fetal ovary restores the normal ovarian phenotype. The results demonstrate that Wnt4 stimulates β-catenin to ensure the proper occurrence of ovarian development during embryogenesis.
4.2 Introduction

Although ovarian development is thought to be a default process (only arises in the absence of Y-chromosome or Sry gene), genetic screening and microarray analyses have identified many genes that express specifically in the fetal ovary. These ovary-specific genes include *Dax1* (49), *Foxl2* (21, 50), follistatin (51, 52), *Wnt4*, (53), *Wnt5a, Wnt6a, Wnt9a*, (54), and R-spondin 1 (24, 55, 56). These genes are believed to be involved in ovarian development. The most interesting group of genes among these ovary-specific genes are the Wnt proteins and the associated factors. The WNT pathway plays an essential role in the process of embryonic development (127-129). To elicit their functions, WNT proteins need to bind to their Frizzled receptor and LRP5/6 co-receptor on the cell membrane and transmit intracellular signals via at least three different intracellular pathways, the WNT/polarity pathway, the WNT/Ca^{2+} pathway, and the WNT/β-catenin pathway, depending on the tissue context (130, 200). In addition to WNT proteins, R-spondin, an orphan ligand family, also has been shown to induce WNT/β-catenin signaling pathways and promote the function of WNT signaling by stabilizing the cytoplasmic β-catenin (61, 201, 202). Multiple Wnts and related genes are expressed specifically in the fetal ovary, suggesting that they play a potential role in regulating the development of the fetal ovary.

In mice, removal of either *Rspo1* or *Wnt4* led to partial female-to-male sex reversal with appearance of testis vasculature, ectopic adrenal derived androgen-producing cells, and maintenance of the androgen-dependent Wolffian duct, the male reproductive tract precursor (53, 55, 56). RSPO1 and WNT4 proteins, both secreted factors, are known to induce a cellular response via β-catenin (61). In Chapter 2, I have demonstrated that inactivation of β-catenin in the somatic cells of the fetal ovary results in the appearance of testis vasculature, ectopic appearance of male-specific characteristics, and the loss of female germ cells in the fetal ovary. These phenotypes are very similar to the ones observed in the *Wnt4* and *Rspo1* null mutation mice, indicating these three factors are in the same pathway to regulate fetal ovary development. Furthermore, the results of in situ hybridization revealed that *Wnt4* mRNA was absent in the absence of β-catenin in the somatic cells of fetal ovary, while *Rspo1* mRNA was still present. These data indicate that *Wnt4* is downstream of β-catenin. However, the possibility that WNT4 also signals through β-catenin in regulating the development of the fetal ovary cannot be ruled out based on this evidence. It is possible that WNT4 regulate its own expression via β-catenin. Therefore to further investigate the molecular composition between WNT4 and β-catenin, I
applied a genetic approach that ectopically activates β-catenin in the somatic cells of the Wnt4 KO fetal ovary. If WNT4 signals via β-catenin, I expect to observe that the activation of β-catenin in the Wnt4 KO background female will rescue the abnormal phenotypes.

4.3 Experimental procedures

4.3.1 Animals

The Wnt4+/− mice were obtained from Jackson Laboratory (strain 129-Wnt4<sup>tm1Amc</sup>/J) and the Ctnnb1<sup>β(ex3)</sup> mice were from Harada et al. (203). There are three cross steps to generate mice expressing the stabilized form of β-catenin specifically in the somatic cells of the Wnt4 knockout ovary. The first cross was to mate Wnt4 heterozygotes with SF-1/cre mice to obtain Wnt4<sup>+/−</sup>; SF-1/cre (Cross1, see description below). The second cross was to generate Wnt4<sup>+/−</sup>; Ctnnb1<sup>β(ex3)</sup> by mating Wnt4 heterozygote with Ctnnb1<sup>β(ex3)</sup> homozygote mice (Cross2).

Lastly, the Wnt4<sup>+/−</sup>; SF-1/cre were mated with Wnt4<sup>+/−</sup>; Ctnnb1<sup>β(ex3)</sup> mice to obtain Wnt4<sup>−/−</sup>; Sf-1cre; Ctnnb1<sup>β(ex3)</sup> animals (Cross3). The chance to obtain a Wnt4<sup>−/−</sup>; Sf-1cre; Ctnnb1<sup>β(ex3)</sup> female is about one out of thirty-two.

The Cross Scheme:

Cross 1. Wnt4<sup>+/−</sup> X SF-1/cre → Wnt4<sup>+/−</sup>; SF-1/cre

Cross 2. Wnt4<sup>+/−</sup> X Ctnnb1<sup>β(ex3)</sup> → Wnt4<sup>+/−</sup>; Ctnnb1<sup>β(ex3)</sup>

Cross 3. Wnt4<sup>+/−</sup>; SF-1/cre X Wnt4<sup>+/−</sup>; Ctnnb1<sup>β(ex3)</sup> → Wnt4<sup>−/−</sup>; Sf-1cre; Ctnnb1<sup>β(ex3)</sup>

Female and male mice were housed together and females were checked for the presence of a vaginal plug the next morning. The day when the vaginal plug was detected was considered 0.5 day of gestation, or 0.5 day post coitum (0.5 dpc).

4.3.2 Genotyping

For genotyping, tail clippings were digested in 200 µl of 50 mM sodium hydroxide for 30-45 minutes at 95°C, then 100 µl of 1 M Tris HCl, pH=8 was added and mixed before centrifugation at 10,000 g for 5 minutes at room temperature. Samples were then subjected to standard PCR using gene-specific primers. A 25 µl standard PCR reaction was set up as
following: 1 µl of tail DNA, 12.5 µl of 2X PCR Mango Mix (Bioline), 1.25 µl of 10 mM gene-specific primers and 10.25 µl of double distilled water. All PCR reactions were run using the BIO-RAD MyCycler Thermal Cycler followed by running on 1.5 % or 2% TAE gel at 100 volts. The primers and PCR reactions for genotyping were listed in Table 4.1 and Appendix A.

4.3.3 Immunohistochemistry

Samples were fixed in 4% paraformaldehyde overnight at 4 °C and then washed in PBS for 5 minutes (3 times). Samples were put through a sucrose gradient (10%, 15% and 20 %) and incubated in 1:1 20% sucrose and OCT freezing media (Tissue-Tek) overnight at 4 °C. Samples were embedded in 1:3 20% sucrose and OCT mix and cut to 10 µm thick frozen sections. Sections were washed with PBS and then blocked in the blocking solution (5% heat-inactivated donkey serum and 0.1% Triton X-100 in PBS) for 1 hour at room temperature. Primary antibodies were added to the blocking solution and incubated with sections at 4 °C overnight. Sections were then washed with the washing solution (1% heat inactivated donkey serum and 0.1% Triton X-100 in PBS) followed by incubation in the blocking solution with the corresponding secondary anybodies. Sections were then washed with the washing solution and mounted with DAPI antifade reagent. The sources and dilution of primary antibodies were: rat monoclonal antibody against germ cell nuclear fraction (TRA98, 1:000, a gift from H. Tanaka), the rat polyclonal antibody against PECAM-1 (1:500; Pharmingen), and rabbit polyclonal antibody against CYP17 (1:100, a gift from B. Hales). All the secondary antibodies were purchased from Jackson Immunochemical and a 1:200 dilution was used.

4.3.4 Germ cell counting

Newborn ovaries were obtained from 3 animals for each genotype. Samples were fixed in 4% paraformaldehyde in PBS at 4°C and processed according to the immunohistochemistry procedure described above. Germ cell count was obtained by counting TRA98-positive germ cells in all sections (30 µm apart) from an entire ovary. Data were analyzed using one-way ANOVA followed by Tukey test for pairwise comparisons.

4.3.5 Transformation and mini preparation of plasmid

One µl of plasmid solution was added to 50 µl of DH5α competent cells and then placed
on ice for 30 minutes. The tube was subjected to heat shock at 42°C for 40 seconds. 1ml of Luria-broth (LB) or SOC media was added to the tube followed by incubation at 37°C for 1 hour. Bacteria solution was centrifuged at 2000 g for 5 minutes at room temperature. The 950 µl supernatant was discarded and bacteria were suspended in the remaining media (~100 µl) by pipeting. Next, 100 µl of the sample were placed on a Luria-broth ampicillin agar plate and incubated overnight at 37°C. The tip used to spot each of the colonies was dropped into a separate tube of 3 mL LB with ampicillin and incubated at 37°C with shaking for 16 hours. The Qiagen mini-prep kit was used for DNA preparation (Cat. no. 27106, Qiagen, USA). In brief, the bacteria solution was collected in 1.5 ml tubes. The tubes were centrifuged at maximum speed for 1 minute and the supernatant was removed. Then 250 µl of cold buffer P1 were added to the solution and the pellet was suspended using vortexing. Next, 250 µl of buffer P2 were added in the tube and mixed with buffer P1 thoroughly by inverting the tube 4–6 times incubated at room temperature. Then 350 µl of buffer N3 were added and mixed immediately and thoroughly by inverting the tube 4–6 times. Samples then were centrifuged at 10,000 g for 10 minutes at room temperature and the supernatant was transferred to the QIAprep spin column followed by washing with buffer PB and buffer PE. Finally, DNA was eluted from the column by adding TE buffer.

4.3.6 RNA probe synthesis

Five µg of plasmid were treated with the appropriate restriction enzyme for linearization then purified by mixing with 1 volume phenol/ chloroform /isoamyl alcohol (25:24:1) and centrifuging at 10,000 g for 10 minutes. The supernatant was moved to a new tube and the residual phenol was removed by one volume of chloroform/isoamyl alcohol (24:1). DNA was precipitated by adding 2 volumes of 100% ethanol and incubated at -20 °C for at least 2 hours. DNA was then recovered by centrifugation at 10,000 g, 4 °C for 30 minutes and washed with ice-cold 70 % ethanol. The in vitro transcription reaction contained: 1 µg of linear plasmid, 2 µl of 10X transcription reaction buffer, 2 µl of Digoxigenin nucleotide mix, 1 µl of appropriate RNA Polymerase, and DEPC-water to a volume of 20 uL. Samples were incubated at 37°C for 2 hours. Then 0.8 µl of 0.5M EDTA were added to stop the reaction. Next, 1 µl of 10M Lithium chloride and 75 µl cold 100% ethanol was added to the reaction mixture. Samples were placed at -20°C for at least 2 hours followed by centrifugation at 10,000 g speed for 30 minutes at 4°C.
After the centrifugation, the supernatant was removed and the pellet was washed with ice cold 75% DEPC-ethanol and re-suspended in 20 µl DEPC water. Finally, the probe was diluted 1:1 with hybridization buffer (5x SSC pH 5.0, 50% formamide, 0.1% CHAPS, 0.1% Tween20, 1mg/ml Yeast tRNA, 50 µg/ml Heparin, and 5mM EDTA pH 8.0) and stored at -20 °C. A gel check was also utilized to determine if the probe was transcribed correctly.

4.3.7 Whole-mount in situ hybridization

Tissues were fixed overnight in 4% paraformaldehyde in PBS at 4°C and dehydrated through a methanol gradient (25%, 50%, 70%, and 100%) in PTW (0.1% Tween20 in DEPC-PBS). Samples were stored in 100% methanol at -20°C. In situ hybridization was processed according to the standard non-radioisotopic procedure using digoxigenin-labeled RNA probes. In brief, samples were dehydrated through a methanol gradient then treated with proteinase K (10 mg/ml) at 37°C for 12 minutes followed by fixation in 4% paraformaldehyde/0.1% glutaldehyde immediately at room temperature for 20 minutes. Samples were pre-hybridized in hybridization buffer (5x SSC pH 5.0, 50% formamide, 0.1% CHAPS, 0.1% Tween20, 1mg/ml Yeast tRNA, 50 µg/ml Heparin, and 5mM EDTA pH 8.0) at 65 °C for 1 hour. Then digoxigenin-labeled RNA probe was added into the solution and samples were rotated in an oven at 65°C overnight (12-16 hours). Samples were washed the next day in pre-warmed hybridization buffer followed by MABTL (5% MAB, 0.1% Tween20 and 0.05% Levamisol). Samples were incubated in 20% sheep serum in MABTL blocking solution at room temperature for 2 hours followed by incubation in alkaline phosphatase-conjugated anti-digoxigenin at 4°C on shaker overnight. On the third day, after washing in MABTL three times for 1 hour each, samples were incubated in alkaline phosphates substrate in NTMTL (0.1 M NaCl, 0.01 M Tris-HCl pH 9.5, 0.05 M MgCl2, 1% Tween20, 0.05% Levamisol) for color development. The optimal hybridization temperature and sources of RNA probes are: Fst (65 °C; Martin Matzuk), and Inhbb (65 °C; Martin Matzuk).

4.3.8 Chromatin immnuoprecipitation assay

Sixty to seventy gonads without mesonephroi attached were collected from 13.5 dpc embryos. These gonads were pooled in 1 ml cold-PBS. Chromatin was cross-linked in 1% formaldehyde at 37°C for 15 minutes. Gonads were then washed (three times) with 1x proteinase inhibitor in 1ml cold-PBS at 4°C for 10 minutes and stored at -80°C. Once the sample was ready,
the ChIP assay was performed using EzChIP kit (Millipore, Cat no17-371). Chromatin was sonicated to shear the DNA by using Dismembrator Model 100 (Fisher Scientific) at power 3 for 3X10 seconds (wait for 1 minute to cool down the sample between each sonication) on wet ice. The sonicated chromatin was diluted in ChIP dilution buffer. Protein G agarose (60 µl) was added to prevent non-specific binding. The input sample is 1% of the pre-cleared supernatant. Immunoprecipitation was performed with 4 µg anti-β-catenin antibody (Millipore, Cat no 06-734), 1 µg anti-RNA polymerase II or IgG at shaken at 4 °C overnight. To precipitate the chromatin-protein complex, 60 µl protein G agarose were added to the sample. Next, the sample was washed using different buffers provided in the kit (Low salt wash buffer, high salt wash buffer, LiCl wash buffer and TE buffer). The chromatin then eluted from antibody/agarose complex by adding elution buffer. The eluded chromatin then mixed with 5 M NaCl and incubated at 65 °C for at least 5 hours to reverse cross-linkage. The next step was to add 10 µl of 0.5 M EDTA, 20 µl of 1 M Tris-HCl, and 2 µl proteinase K followed by incubation for 1 hour. Finally, DNA was purified using spin columns. PCR reaction with specific primers was performed on the prepared sample. The primer sequences used for PCR reaction were listed in Table 4.2 and Appendix A.

4.3.9 Reverse Transciptase (RT) and RT-PCR

Gonads of CD-1 mice from 12.5 dpc embryos or gonads of Ctnnb1fl(ex3/+); Ctnnb1fl(ex3/+), and Sf1cre; Ctnnb1fl(ex3/+), from 13.5 dpc embryos were separated from mesonephros, snap frozen and stored at -80 °C for further processing. For RT-PCR, 10-15 frozen 12.5 dpc gonad were processed with TRIzol reagent for total RNA extraction (Cat. no. 15596-026, Invigrogen). RNA was treated with RQ1 RNase-Free DNase (Cat. no. M6101, Promega) For real time quantitative PCR (Q-PCR), a single pairs of frozen 13.5 dpc gonad were processed with Qiagen RNAeasy kit for total RNA extraction (Cat. no. 74104 Qiagen) cDNA libraries were created using Omniscript RT kit (Cat. no. 205113 Qiagen) according to the manufacturer’s protocol. Q-PCR was performed in triplicate using SsoFast™ EvaGreen® supermix (Cat. no. 172-5203, Bio0Rad) and run on the CFX96 real-time PCR system. The primers used for RT-PCR and Q-PCR reactions are listed in table 4.3, 4.4 and Appendix A.
4.4 Results

4.4.1 Expression of Fst is restored upon the activation of β-catenin in the somatic cells of Wnt4 knockout ovary

The expression of Wnt4 was absent in the β-catenin conditional knockout (cKO) ovary, placing Wnt4 in the downstream of β-catenin. However, WNT4, as a secreted factor, might also act on the somatic cells of the fetal ovary and further stimulate β-catenin in regulating the development of the fetal ovary. To examine whether β-catenin serves in the downstream of Wnt4 to regulate ovary development, I investigated whether ectopic activation of β-catenin was able to rescue the phenotypes in the Wnt4 knockout ovaries.

To achieve this goal, I introduced a constitutively active form of β-catenin (Ctnnb1fl.(ex3)) specifically in the SF1-positive somatic cells in the Wnt4 KO ovary. Ctnnb1fl.(ex3) mice contain a genetically engineered β-catenin gene that has loxP sites inserted in either side of exon 3 (203). The peptides encoded by exon 3 are responsible for degradation of β-catenin. Once exon 3 is removed by the Cre recombinase, β-catenin becomes resistant to degradation and therefore constitutively active in the SF1-positive somatic cells.

Follistatin, an activin binding protein, is an ovary-specific factor and is also known to regulate the hypothalamic-pituitary-gonadal axis (60). In the absence of Wnt4 or β-catenin, the expression of Fst was also absent in the fetal ovary. Thus, if WNT4 signals through β-catenin to regulate the expression of Fst, ectopic expression of β-catenin in the somatic cells of Wnt4 KO ovary should restore the expression of Fst. To test this hypothesis, I applied in situ hybridization on 13.5 dpc ovaries. As expected, the expression of Fst was present in the control ovary but was absent in the Wnt4 KO ovary (Fig. 4.1 A & B, n=1). However, the expression of Fst was observed in the somatic cells of Wnt4 KO ovary with re-activated β-catenin (Wnt4−/−;Sf-1cre; Ctnnb1fl(ex3), Fig. 4.1 C, n=1). These data demonstrated that WNT4 regulated follistatin via β-catenin in the fetal ovary.

4.4.2 Coelomic vessel is thinner in the Wnt4 knockout with activated β-catenin ovary than in the Wnt4 knockout ovary

Next, I analyzed whether activation of β-catenin in the Wnt4 KO ovary rescued the presence of testis-specific characteristics and the abnormal masculinization in the Wnt4 KO
ovary. I first investigated whether the testis-specific vasculature, the coelomic vessel, was still ectopically present or not. By applying immunostaining with PECAM-1 antibody, which marked endothelial cells of blood vessels and primordial germ cells in the gonads, the coelomic vessel was found in the control testis and Wnt4 KO ovary but not in the control ovary (Fig. 4.2 A-C). However, coelomic vessel was still present in the Wnt4−/−; Sf-1cre; Ctnnb1fl(ex3) ovary (Fig. 4.2 D), suggesting that WNT4 may not signal via β-catenin to prevent the formation of coelomic vessel in the fetal ovary or the ectopic activation of β-catenin by using SF-1/cre may be too late to prevent the appearance of coelomic vessel. In addition, the coelomic vessel in the Wnt4−/−; Sf-1cre; Ctnnb1fl(ex3) ovary did not form properly and the vessel was much thinner than the one in the Wnt4 KO, indicating that the ectopic activation of β-catenin in the Wnt4 KO ovary affected the formation of coelomic vessel.

### 4.4.3 Expression of Inhbb is down-regulated in the Wnt4 knockout ovary with activation of β-catenin.

The coelomic vessel in the Wnt4−/−; Sf-1cre; Ctnnb1fl(ex3) ovary did not form properly, suggesting that the activation of β-catenin may interfere the process of vessel formation. Previous studies indicate that inhibin βb (Inhbb) is responsible for the formation of coelomic vessel in the Wnt4 KO ovary (59). In Chapter 2, I also demonstrated that the coelomic vessel appeared in the ovary with absence of β-catenin and the expression of Inhbb was up-regulated. Therefore, to investigate whether activation of β-catenin in the somatic cells of Wnt4 knockout ovary affects the expression of Inhbb, I performed in situ hybridization with Inhbb probe at 13.5 dpc gonads. The expression of Inhbb was maintained at a low level in the control ovary but was increased dramatically in the Wnt4 KO ovary (Fig. 4.3 A & B, n=2). Introduction of active β-catenin to the Wnt4 KO ovary reduced the expression of Inhbb to the level similar to that in the control. The results indicate that WNT4 signals through β-catenin to regulate the expression of Inhbb in the fetal ovary.
4.4.4 Activation of β-catenin in the somatic cells of Wnt4 knockout ovary prevents the ectopic appearance of androgen-producing cells and rescues the female from masculinization

Next, I investigated whether activation of β-catenin in the Wnt4 KO ovary rescued the abnormal masculinization in the Wnt4 KO ovary. The ectopic appearance of androgen-producing cells was found in the Wnt4 KO and β-catenin cKO ovaries. By applying immunostaining with CYP17 antibody, an androgen-producing enzyme, the CYP17-positive cells were only present in the control testis but not in the control ovary (Fig. 4.4 A & B). However, the ectopic CYP17-positive cells in the Wnt4 KO ovary (Fig. 4.4 C) were no longer present in the Wnt4−/−;Sf-1cre; Ctnnb1fl(ex3) ovary (Fig. 4.4 D), indicating that activation of β-catenin in the SF-1-positive Wnt4 KO somatic cells was able to prevent the ectopic appearance of CYP17-positive cells. The ectopic appearance of androgen-producing cells was responsible for the maintenance of epididymis in the Wnt4 KO and β-catenin cKO females. Based on the results that these CYP17-positive cells disappeared in the Wnt4−/−;Sf-1cre; Ctnnb1fl(ex3) ovary, I expected that the epididymis would not be maintained in the Wnt4−/−;Sf-1cre; Ctnnb1fl(ex3) female. As predicted, in the absence of CYP17-positive androgen-producing cells, epididymes disappeared in the Wnt4−/−;Sf-1cre; Ctnnb1fl(ex3) female (Fig. 4.5 B & D). In addition, WNT4 also regulates the development of the Müllerian duct, which will develop into the female reproductive tract later. Thus, in the absence of Wnt4, the female reproductive tract was degenerated and this caused the ovary of Wnt4−/−;Sf-1cre; Ctnnb1fl(ex3) to only be connected to the ligament-like structure (Fig. 4.5 D). These results together demonstrated that WNT4 indeed signals through β-catenin to inhibit the ectopic appearance of androgen-producing cells in the fetal ovary that prevented female from masculinization.

4.4.5 Activation of β-catenin in the somatic cells of Wnt4 knockout ovary restores the number of germ cells

Female germ cells were lost in both Wnt4 KO and β-catenin cKO ovaries. To understand whether WNT4 maintains the survival of female germ cells via β-catenin, I examined the development of germ cells in the Wnt4−/−;Sf-1cre; Ctnnb1fl(ex3) fetal ovary by using immunostaining with TRA98 antibody.
As expected, most TRA98-positive female germ cells were lost in the Wnt4 KO ovary at birth (Wnt4−/−; SF1/cre, Fig. 4.6 F) compared to the controls (Wnt4+/−;SF1/cre, Fig. 4.6 E). In contrast, introduction of the active β-catenin to SF1-positive Wnt4 KO somatic cells (Wnt4−/−; SF1/cre; Ctnnb1fl.(ex3), Fig. 4.6 H) resulted in more germ cells present at birth comparing to the Wnt4 KO (Fig. 4.6F). To examine whether activation of β-catenin in the SF1-positive Wnt4 KO somatic cells restores the number of germ cells, I quantified the number of germ cells by determining the TRA98-positive cell counts across all sections (30 µm apart) of an entire ovary. Activation of β-catenin in the SF1-positive somatic cells of the fetal ovary resulted in an increased size of ovary (Fig. 4.6 C & D). The size of ovary in Wnt4+/−;SF1/cre;Ctnnb1fl.(ex3) (Fig. 4.6 C) and Wnt4−/−;SF1/cre;Ctnnb1fl.(ex3) (Fig. 4.6 D) were larger than the ones in Wnt4−/− (Fig. 4.6 B) and control (Fig. 4.6 A). Thus, more sections from the Wnt4+/−;SF1/cre;Ctnnb1fl.(ex3) and Wnt4−/−;SF1/cre;Ctnnb1fl.(ex3) (around 60 and 48 sections, respectively) were examined comparing to the Wnt4−/− and control ovaries (around 36 and 30 sections, respectively). I found that the induction of active β-catenin to SF1-positive Wnt4 KO somatic cells (Wnt4−/−; SF1/cre; Ctnnb1fl.(ex3), Fig. 4.6 H) restored total female germ cell number to a level statistically similar to that in the Wnt4+/−; SF1/cre and Wnt4+/−; SF1/cre; Ctnnb1fl.(ex3) (Fig. 4.6 E & G & I, n=3 embryos for each genotype).

4.4.6 Consistent activation of β-catenin in the fetal ovary causes reduced expression of Wnt4, Fst and Rspo1

The above results demonstrate that WNT4 transmits the cell signals to β-catenin in the SF-1 positive-somatic cells of fetal ovary. I have shown that the expression of Wnt4 was controlled by β-catenin in the fetal ovary in Chapter 2. Therefore, it is possible that WNT4 could stimulate its expression via β-catenin. To test this hypothesis, I performed real time quantitative PCR (Q-PCR) at 13.5 dpc SF1/cre; Ctnnb1fl.(ex3) ovaries in which β-catenin was consistently activated in the SF-1-positive somatic cells. The results showed that the expression of Wnt4, Fst and Rspo1 were all down-regulated in the SF1/cre; Ctnnb1fl.(ex3) (Fig. 4.7, n=3)
4.4.7 β-catenin does not regulate *Fst* via binding the consensus TCF/LEF responding element in the proximal promoter region of *Fst*

In Chapter 2, I have demonstrated that the expression of *Fst* was controlled by β-catenin. However, the results did not distinguish whether β-catenin regulated *Fst* directly or indirectly. To address this, I performed Chromatin immunoprecipitation (ChIP) at 13.5 dpc embryonic gonads using β-catenin antibody. Because β-catenin works with TCF/LEF transcription factor as a co-transcriptional factor, by observing whether this assay pulls down the promoter regions of *Fst* would determine the difference.

To have high efficiency of immunoprecipitation, the cross-linked chromatin was sonicated to the fragment size from 200bp -1kb. A gel running confirmed that the size of the DNA shearing was within the desired range (Fig. 4.8 A).

Using ChIP assay with RNA polymerase II antibody, I observed that the promoter region of *Gapdh* was immunoprecipitated by RNA polymerase II. The result indicated that the ChIP assay worked in my system (Fig. 4.8 B). Furthermore, because Axin2 is known to be the downstream target of β-catenin (204) and is expressed strongly in the XX gonad and mesonephroi (56). Thus, I used Ainx2 as my positive control for the β-catenin ChIP assay. I found the promoter region of Axin2 in the mesonephroi and ovary was immunoprecipitated by β-catenin antibody. In addition, I also observed that the promoter region of Axin2 in the testis was weakly immunoprecipitated by β-catenin (Fig. 4.8 C). Although I did not observe the sexually dimorphic results for Axin2 by applying β-catenin ChIP assay, my results at least demonstrated the β-catenin ChIP assay in embryonic gonads in my system worked. However, no positive band of β-catenin immunoprecipitated chromatin was observed in the testes, ovaries and mesonephroi while applying PCR using specific primers for the proximal region of *Fst* promoter, in which contains a consensus TCF/LEF binding element(141) (Fig. 4.8 D). These results suggested that β-catenin might not regulate the expression of *Fst* via binding this region.

4.4.8 Multiple frizzled receptors are present in the embryonic ovary

To transmit the signals into cytoplasm, WNT4 binds the receptor on the cell membrane, Frizzled (*Fzd*) (142). *Fzd* proteins are seven-transmembrane receptors. WNT proteins can bind the cystein-rich domain of *FZD* (142). A total of ten different *Fzd* receptors have been found in
the rodent. Therefore, to indentify which Fzds are present in the fetal gonads and are the receptors for WNT4, I performed RT-PCR to detect the expression of different Fzds at the mRNA level. An In situ hybridization assay has revealed that Fzd10 is absent in the embryonic gonads (205) and Fzd3 and Fzd9 KO mice do not develop abnormal ovarian phenotypes. Thus, my study focused on investigating whether there was expression of Fzds 1-2 and Fzds 4-8 in the embryonic gonads. I found that Fzd4 and Fzd5 were both present in the embryonic testes and ovaries while Fzd1 and Fzd7 were found in the ovaries at 12.5 dpc (Fig. 4.9, n=2). RNA from adult ovaries was used as a positive control for each Fzd to show the specificity of the PCR products (206).

4.5 Discussion

In Chapters 2 and 3, I have indentified that the WNT4/β-catenin pathway, operating in the SF1-positive somatic cells in fetal ovaries, is essential for maintaining the ovarian identity and supporting the survival of meiotic germ cells. Inactivation of β-catenin in the SF-1 positive somatic cells of the fetal ovary resulted in the loss of Wnt4 mRNA expression, placing WNT4 downstream of β-catenin. In this chapter, I further demonstrated that WNT4 also activated β-catenin in the SF1-positive somatic cells of the fetal ovary to maintain ovarian identity as well as support the survival of female germ cells (Fig 4.11). Activation of β-catenin in the SF1-positive somatic cells of the fetal ovary prevented the ectopic appearance of androgen-producing cells and the masculinization of females and restored the number of germ cells in the Wnt4 KO mice. Evidence of WNT4 signaling via β-catenin was also found in nephron induction, kidney epithelial cells, and renal fibrosis (154, 174, 207, 208). These observations collectively support the model that β-catenin operates downstream of WNT4 in the fetal ovary. In addition to serving as an intracellular signaling molecule of WNT4, β-catenin also has a potential role in regulating the expression of Wnt4. I found that the expression of Wnt4 was lost in the absence of β-catenin in the SF-1 positive-somatic cells of fetal ovary; however, R-spondin 1 (Rspo1) expression was not altered. These results suggest that Rspo1 or other WNT proteins, including WNT4 itself may stimulate Wnt4 expression via β-catenin. The experiment by introducing activated form of β-catenin was also perfomed in the Rspo1 null mutation background mice. The null mutation of Rspo1 ovary develops ovotestis in adulthood. However, if β-catenin is activated in the Rspo1
KO ovary, the ovary was rescued from becoming ovotestis \((56)\). The results were similar to what I observed in the \(\text{Wnt}^{4^{-/}};\text{Sf-1cre};\text{Ctnnb1}^{\beta(ex3)}\), indicating that RSPO1 and WNT4 both activated \(\beta\)-catenin in the ovary development. However, the data could not explain whether RSPO1 or WNT4 stimulate \(\beta\)-catenin alone or if they work with each other to do so.

Surprisingly, the expressions of \(\text{Wnt4}, \text{Fst}\) and \(\text{Rspo1}\) were all reduced in the \(\text{Sf-1cre};\text{Ctnnb1}^{\beta(ex3)}\) ovary at 13.5 dpc (Fig.4.7). This reduction might result from the negative feedback control of WNT pathway. The negative feedback control of WNT pathway is also found in other tissues such as hair follicles and tumor cells \((209, 210)\). The activation of WNT pathway could induce the Wnt inhibitor, e.g., Dickkopf, to attenuate canonical WNT signaling \((210)\). Further examination on the expression of \(\text{Wnt4}, \text{Fst}\) and \(\text{Rspo1}\) at earlier stages, e.g. 12.5 dpc will provide a more conclusive explanation.

The coelomic vessel, a testis-specific vasculature, was still present in the \(\beta\)-catenin activated somatic cells of \(\text{Wnt4} \) KO fetal ovary. This observation suggests that WNT4 may act through different mechanisms in preventing the ectopic appearance of coelomic vessel in the fetal ovary. The endothelial cells, which contribute to the formation of coelomic vessel, migrate from the mesonephros to the XY gonad after sex determination occurs. It has been shown that WNT4 suppresses this migration event and prevents the formation of coelomic vessel in the fetal ovary \((58)\). \(\text{Wnt4}\) starts to express in the gonadal ridge of the mouse around 9.0 dpc in both sexes. Its expression decreases in the testis but is maintained in the ovary at 11.5 dpc. In my study, I utilized the SF-1cre mouse line to activate the stabilized form of \(\beta\)-catenin in the fetal gonad. SF-1cre starts to be functional around 11.5 dpc \((159)\). Therefore, the appearance of coelomic vessel in the \(\text{Wnt}^{4^{-/}};\text{Sf-1cre};\text{Ctnnb1}^{\beta(ex3)}\) fetal ovary may result from the delayed stabilization of \(\beta\)-catenin. In addition, the coelomic vessel of the \(\text{Wnt}^{4^{-/}};\text{Sf-1cre};\text{Ctnnb1}^{\beta(ex3)}\) fetal ovary was much thinner compared to the one of the \(\text{Wnt4} \) KO ovary, indicating that the activation of \(\beta\)-catenin interrupted the formation process of coelomic vessel. In addition, if \(\text{Wnt4} \) XX gonads were cultured with lithium chloride (LiCl), which is an inhibitor of GSK3\(\beta\), to activate the \(\beta\)-catenin pathway by preventing \(\beta\)-catenin from degradation, the \(\text{Wnt4} \) KO XX gonad does not form a coelomic vessel \((172)\). Genetic evidence reveals that \(\text{Inhbb}\), the subunit of activin B or inhibin B, is responsible for the formation of coelomic vessel in the \(\text{Wnt4}\) and \(\text{Fst} \) KO ovaries \((59)\). The elevated expression of \(\text{Inhbb}\) was found both in the absence of \(\text{Wnt4}\) (Fig. 4.3 B and \((59)\)) and \(\beta\)-catenin (Fig. 3.6 C) whereas \(\text{Inhbb}\) expression at normal level was observed in the \(\text{Wnt}^{4^{-/}};\text{Sf}-\)
Icre; Ctnnb1β(ex3) ovary (Fig. 4.3 C). Furthermore, Fst mRNA, which encodes an activin binding protein, was restored in the Wnt4c; Sf-Icre; Ctnnb1β(ex3) fetal ovary (Fig. 4.1). This evidence supports the model that WNT4 activates β-catenin in the somatic cells of the fetal ovary to prevent the ectopic appearance of coelomic vessel.

The expression of mouse Fst requires a consensus LEF/TCF responding element in its proximal prompter region (141, 142). The expression of Fst was lost in the Wnt4 and β-catenin cKO ovaries, placing Fst in the downstream of Wnt4 and β-catenin. Fst mRNA was observed in the Wnt4 KO fetal ovary with ectopic activation of stabilized β-catenin. These results reveal the molecular connection among these three factors: WNT4 acts on the SF-1 positive somatic cells of the fetal ovary to activate β-catenin and to regulate the transcriptional activity of Fst. Inhbb, another gene that was suppressed by β-catenin, has several LEF/TCF responding element in its promoter region. Therefore, it is possible that WNT4 signals through β-catenin to inhibit the expression of Inhbb in the fetal ovary.

In the ChIP assay with β-catenin antibody, I did not observe the positive signal from PCR using specific primers for the proximal region of Fst promoter containing a LEF/TCF responding element. The results suggested that β-catenin did not regulate the expression of Fst via binding this region. However, this result did not rule out the possibility that β-catenin might regulate Fst via other LEF/TCF responding elements that located in the promoter region of Fst (Fig. 4.10) In addition, because the quantity of the immunoprecipitated DNA is often very small (1-10 ng in total), the PCR reaction used in this study might not be sensitive enough to detect it. Further studies with the ligation-mediated PCR (LM-PCR) may improve the sensitivity. Because I only repeated the experiment once (n=2), thus the results presented here are only preliminary.

By using RT-PCR I observed that several Frizzleds were present in the embryonic ovaries, eg. Fzd1, Fzd4, Fzd5, and Fzd7. Previous studies have demonstrated that Fzd1, Fzd5 and Fzd4 interact with WNTs to activate β-catenin (211, 212). These Fzds could be the receptors of WNT4. Studies on follicular development showed that Fzd4 might be the potential receptor for WNT4 in adult ovary because they both co-expressed in the same tissue (206). Although it has been shown that Fzd6 might be the receptor for WNT4 in the regulation of human pituitary adenomas (213), I did not detect the expression of Fzd6 in the fetal ovary. The absence of mRNA expression of Fzd6 in the fetal gonads by using RT-PCR was consistent with the results of in situ hybridization (205). This evidence indicates that WNT4 works with other FZD in the
fetal ovary. Although the results of RT-PCR revealed that \textit{Fzd1}, \textit{Fzd4}, \textit{Fzd5}, and \textit{Fzd7} were expressed in the embryonic ovary, the data could not explain the location of these Fzds in the fetal ovary. These Fzds could be either present on the somatic cells or germ cells or both. Further experiments to identify the localization of these Fzds in the fetal ovary would be informative.

In summary, in addition to being regulated by $\beta$-catenin, WNT4 also signals via $\beta$-catenin, an intracellular factors, to ensure the proper ovarian development (Fig.4.11). The present study has provided genetic evidence that WNT4 signals via $\beta$-catenin in the SF1-positive somatic cells of the fetal ovary.
### 4.6 Tables and figures

<table>
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<th>Target gene</th>
<th>Forward primer-5'</th>
<th>Reverse primer-3'</th>
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<tr>
<td>Ctnnb1&lt;sup&gt;fl(ex3)&lt;/sup&gt; floxed allele</td>
<td>GGTAGTGTTCCCTGCCCTTGACAC</td>
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<td>Ctnnb1&lt;sup&gt;fl(ex3)&lt;/sup&gt; WT allele</td>
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<tr>
<td>SF/1Cre</td>
<td>GTGTGAACGAACCTCGTCA</td>
<td>GCATTACGGTGCGATGCAACGATGAT</td>
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<tr>
<td>Sry</td>
<td>TGAAGCTTTTGGCTTTTGAG</td>
<td>CCGCTGCAAATCTCTTTG</td>
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<td>Wnt4 WT allele and null allele</td>
<td>CTG AGGAGACAGGGTAC</td>
<td>1. ATGGTCAACC CCCATTTTACA</td>
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**Table 4.1 Primer sequences for genotyping**
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<tr>
<th>Target gene</th>
<th>Sequence</th>
<th>Detected region</th>
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| *Gapdh*    | FW: AAATGAGGGCGGTCAAAAGAGA  
             RE: AGGGAGGGCTGACGTCCGTTTTAT | The proximal region of *Gapdh* promoter |
| *Axin2*    | FW: TGCTCTCTGTAAGGGGTAGGT  
             RE: TTGAAGTTAGCAGTGGAAGGCTGC | The LEF/TCF responding element within Intron 1 of *Axin2* |
| *Fst*      | FW: TGCAGGACTGATGGATTTCTCCCT  
             RE: AGGTGACATTTCCACCTTGGGA | The LEF/TCF responding element within -500 upstream of proximal promoter region of *Fst* |

**Table 4.2 primer sets for ChIP assay**
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<td>Frizzled4</td>
<td>CTGGGCACCTCTTTGCATATTCTGC</td>
<td>TCAAAGCTGTGACCTGCTAAGTG</td>
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<td>Frizzled5</td>
<td>GACGCCGAGGTCTGTGTAT</td>
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<td>Frizzled6</td>
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<tr>
<td>Frizzled7</td>
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<td>CACTCCAGGTAAAGCTCAGTG</td>
<td>345</td>
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<tr>
<td>Frizzled8</td>
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Table 4.3 primer sets for RT-PCR
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<td>Fst</td>
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<td>GGTCACACAGTAGGCATTATGGTC</td>
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<td>Rspo1</td>
<td>GTCTATCTTGGGGGTGGTTC</td>
<td>AGGGGTGCTCTTTGCTAA</td>
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Table 4.4 primer sets for Q-PCR
Fig. 4.1 Effects of activation of β-catenin in the somatic cell of *Wnt4* KO ovary on the expression of *Fst*. Whole mount *in situ* hybridization with *Fst* probe on 13.5 dpc in control (A) *Wnt4* KO (B) and *Wnt4*^−/−; *SF1/cre; Ctnnb1^fl.(ex3)* (C) ovary. O= ovary; m= mesonephros. Scale bar= 250 mm.
Fig. 4.2 Effects of activation of β-catenin in the somatic cell of Wnt4 KO ovary on the formation of the coelomic vessel. Immunohistochemistry for PECAM-1, a endothelial cell and primordial germ cell marker in control testis (Wnt4<sup>+/−</sup>; SF1/cre) (A) Control ovary (Wnt4<sup>+/−</sup>; SF1/cre) (B) Wnt4 KO ovary (Wnt4<sup>−/−</sup>; SF1/cre) (C) and Wnt4<sup>−/−</sup>; SF1/cre; Ctnnb1<sup>fl.(ex3)</sup> ovary (D). White arrow indicates the testis-specific vasculature, coelomic vessel. Scale bar= 500 mm.
Fig. 4.3 Effects of activation of β-catenin in the somatic cell of Wnt4 KO ovary on the expression of Inhbb. (A-C) Whole mount in situ hybridization with inhbb probe on 13.5 dpc in control (A) Wnt4 KO (B) and Wnt4<sup>−−</sup>; SF1/cre; Ctnnb1<sup>fl.(ex3)</sup>(C) ovary. O= ovary; m= mesonephros. Scale bar= 250mm.
Fig. 4.4 Effects of activation of β-catenin in the somatic cell of Wnt4 KO ovary on the ectopic appearance of androgen-producing cells. (A-D) Immunohistochemical staining for androgen-producing enzyme CYP17 in control testis (Wnt4+/−; SF1/cre) (A) Control ovary (Wnt4+/−; SF1/cre) (B) Wnt4−/−; SF1/cre ovary (Wnt4 KO) (C) and Wnt4−/−; SF1/cre; Ctnnb1fl.(ex3) ovary (D). The inset in C represents a higher magnification of the CYP17-positive cells. Scale bar= 100 mm.
Fig. 4.5 Development of the internal reproductive organs in $Wnt4^{-/-}; SF1/cre$ ($Wnt4$ KO) and $Wnt4^{-/-}; SF1/cre; Ctnnb1^{fl.(ex3)}$ at birth. (A-B) are whole-mount light field microscopic images of the urogenital system. (C-D) are higher magnification images of the reproductive tracts and ovaries in A & B, respectively. Epi= epididymis; k= kidney; o= ovary. Scale bar= 500 mm
Fig 4.6 Effects of constitutively active form of β-catenin on female germ cell survival in the Wnt4 KO ovary. (A-D) Whole mount light field images of Wnt4^{+/+};SF1/cre, Wnt4^{+/+};SF1/cre, Wnt4^{−/−};SF1/cre; Ctnnb1^{fl.(ex3)}, and Wnt4^{−/−};SF1/cre; Ctnnb1^{fl.(ex3)} ovaries. (E-H) Immunostaining for germ cell marker TRA98 was performed on Wnt4^{+/+};SF1/cre, Wnt4^{−/−};SF1/cre, Wnt4^{−/−};SF1/cre; Ctnnb1^{fl.(ex3)}, and Wnt4^{−/−};SF1/cre; Ctnnb1^{fl.(ex3)} ovaries at birth. Scale bar=100 µm. (I) Mean (+/− SE) germ cell count was obtained from Wnt4^{+/+};SF1/cre, Wnt4^{−/−};SF1/cre, Wnt4^{−/−};SF1/cre; Ctnnb1^{fl.(ex3)}, and Wnt4^{−/−};SF1/cre; Ctnnb1^{fl.(ex3)} ovaries. Germ cell count was obtained by counting TRA98-positive germ cells in sections (30 µm apart) from the entire ovary at birth (n=3 embryos for each genotype). Tukey tests revealed that the mean germ cell count in Wnt4^{−/−};SF1/cre was significantly different from the mean in Wnt4^{+/+};SF1/cre (P=0.017), Wnt4^{−/−};SF1/cre; Ctnnb1^{fl.(ex3)} (P=0.002) and in Wnt4^{−/−};SF1/cre; Ctnnb1^{fl.(ex3)} (P=0.023). The asterisk represents statistical significance. Scale bar=100 mm
Fig. 4.7 Q-PCR analysis of *Wnt4* *Fst* and *Rspo1* in 13.5 dpc *SF1/cre; Ctnnb1*<sup>flu</sup>(ex3) ovary versus control ovary. All results were normalized to 36B4. Q-PCR were performed triplicate and Error bar represents the standard deviation of thee animals.
Fig. 4.8 The preliminary results of chromatin immuno precipitation (ChIP) assay with RNA Polymerase II antibody and β-catenin antibody. (A) The condition of DNA shearing. (B) ChIP assay with either RNA polymerase II antibody or Normal Rabbit IgG was performed on 13.5 dpc testes, ovaries, and mesonephroi followed by PCR using specific primers for the Gapdh promoter. (C-D) ChIP assay with either β-catenin antibody or Normal Rabbit IgG was performed on 13.5 dpc testes, ovaries, and mesonephroi followed by PCR using specific primers for the Axin2 promoter (C) and for the Fst promoter (D). T= testis, O= ovaries, M= mesonephroi, I= Normal Rabbit IgG, B= negative control. Input DNA was from the genomic chromatin DNA before the immuno precipitation.
**Fig. 4.9 Expression of $Fzd$ receptors in the gonads at 12.5 dpc.** Reverse transcriptase PCR was performed on RNA isolated from 12.5 dpc gonads using primers for Fzds and an internal control, 36B4. T=testis, O=Ovary, AO= adult ovary.
Fig. 4.10 The putative LEF/TCF response elements in the promoter region of Fst. Highlight sequence denotes scores of a perfect match to a LEF/TCF consensus string was higher than 10. \( G \) indicates the transcription start site; \( ATG \) indicates the translation start site.
Fig. 4.11 A proposed model for the somatic cell-derived pathway on development of female ovary and female germ cell survival. β-catenin serves in downstream of WNT4 in the SF-1 positive somatic cells of fetal ovary to suppress the formation of coelomic vessel, prevent the ectopic production of androgen and support the survival of female germ cells. β-catenin inhibits the expression of activin β B(Inhbb), which causes loss of female germ cells and formation of coelomic vessel. β-catenin also stimulates the expression of follistatin (Fst), which acts to antagonize the activity of Activin B, the product of Inhbb. WNT4 may regulate itself via β-catenin.
Chapter 5: Conclusions and Future Directions

In summary, the present studies were conducted to understand the functional role of β-catenin in the development of fetal ovary and female germ cells. Although canonical WNT/β-catenin pathway has been implicated to be involved in the ovary development, only circumstantial evidence has been shown. My research has provided the first genetic evidence to directly prove that the canonical β-catenin plays an essential role in the fetal ovary. In the absence of WNT4/β-catenin pathway from the somatic cells of fetal ovary causes the loss of ovarian identities and the death of female germ cells. The following conclusions are drawn from my studies:

1. **β-catenin pathway is essential for the maintenance of ovarian identity by preventing the ectopic appearance of male-specific characteristics**

   β-catenin conditional knockout (cKO) female mice showed the ectopic appearance of testis-specific characteristics, including the formation of the testis-specific vessel and masculinization. *In situ* hybridization showed the expressions of ovary-specific genes, e.g. *Fst* and *Wnt4*, were abolished in the β-catenin cKO ovary. Based on these results, I conclude that β-catenin is essential for the maintenance of ovarian identity.

2. **β-catenin pathway is dispensable for the development of fetal testis**

   The expressions of SOX9 and AMH, fetal Sertoli cell markers, were present in the β-catenin cKO testis, indicating the development of Sertoli cells was normal. Morphology and immunohistochemistry examination revealed that the testis cords and testis-specific vasculature also developed normally. Furthermore, the male reproductive system was intact and testis morphology was normal in the β-catenin cKO testis. Thus, the data demonstrated that the absence of β-catenin in the SF1-positive somatic cells did not affect the development of fetal testes.

3. **β-catenin participates with RSPO1 to regulate the expression of Wnt4 in the fetal ovary**

   In the absence of β-catenin, *Rspo1* or *Wnt4*, female mice all developed similar defects, e.g. the formation of testis-specific vessel, masculinization, and the loss of germ cells. These
observations indicate that these three factors may act in the same pathway to regulate the development of fetal ovary. In addition, in the absence of β-catenin, the mRNA of Wnt4 was abolished in the fetal ovary but Rspo1 was unaltered, placing β-catenin in between RSPO1 and WNT4.

4. β-catenin also serves in the downstream of WNT4 in the somatic cells of fetal ovary

The ectopic production of androgen, masculinization, and the loss of female germ cells were no longer present in the Wnt4 KO after activating β-catenin in female mice. These data indicated that β-catenin served in the downstream of WNT4 in the somatic cells of fetal ovary to regulate fetal ovary and female germ cells development.

5. β-catenin pathway is required for supporting the development of meiotic female germ cells

In the absence of β-catenin in the SF1-positive somatic cells of the fetal ovary in mice, the number of female germ cells was severely decreased at birth. This decrease in female germ cells started at 16.5 dpc. Despite the germ cell lost, the female germ cells in the β-catenin cKO ovary still initiated the meiosis and proceeded to the pachytene stage of meiosis, indicating the initiation of meiosis was not affected by the absence of β-catenin signaling from the somatic cells.

6. Maintenance of female germ cells hinges upon a delicate balance between positive (WNT4 and β-catenin) and negative (inhibin βB) regulators derived from the somatic cells in the fetal ovary

In addition to germ cell loss, ectopic androgen production and up-regulated inhibin β B (Inhbb) expression were found in the fetal ovary without Wnt4 or β-catenin. After treating the pregnant female mice carrying the β-catenin cKO embryos with an anti-androgen receptor drug, flutamide, I still observed germ cell loss in the β-catenin cKO ovaries. The results indicated that androgens were not responsible for the loss of germ cells. In the Inhbb and Wnt4 double knockout ovaries, the number of germ cell was restored, indicating that the loss of germ cells occurred in the Wnt4 KO or β-catenin cKO ovary was due to the elevated Inhbb. WNT4/ β-
catenin signaling pathway maintains female germ cell survival by inhibiting Inhbb expression in the somatic cells.

Based on the above conclusions, I propose the following model: In the fetal ovary, RSPO1 works with WNT4 to act upon the SF1-positive somatic cells to activate β-catenin. The activated β-catenin prevents the formation of testis-specific vessel, coelomic vessel, and supports female germ cell survival via suppressing the expression of Inhbb. Fst is also controlled by the WNT4/β-catenin signaling pathway. The function of FST is to antagonize the residual of activin B, the protein product of Inhbb, and thus is also important in supporting the survival of female germ cells and preventing the formation of coelomic vessel in the fetal ovary. WNT4/β-catenin signaling pathway also inhibits the appearance of ectopic androgen-producing adrenal-like cells. Finally, WNT4 might also regulate its own expression via β-catenin.

Up to date, several human patients with WNT4 or RSPO1 mutation have been reported (24, 57, 214). The majority of these patients suffer form infertility and/or sex reversal. Although mouse model was used, this research provides the important insights about premature ovarian failures and infertility problems in humans by revealing the cellular components and the signaling pathways required for the ovarian development and maintenance of its identity. My research has unveiled an underlying mechanism involved in the development of fetal ovary.

To further uncover other potential mechanisms in regulating ovarian development, the following experiments are proposed:
1. How does upregulation of *Inhbb* in the absence of WNT4/β-catenin signaling cause the death of female germ cells?

In Chapter 3, I have demonstrated that the elevated expression of *Inhbb* was responsible for the death of female germ cells in the *Wnt4* KO and β-catenin cKO ovaries. The remaining question is whether the *Inhbb* causes the death of germ cells directly or through other mechanisms. Inhibin β B gene encodes the subunits of activin B or inhibin B. Inhibins are known to antagonize activin action extracellularly and do not induce signaling pathways themselves. On the other hand, activins, members of the TGF-β family proteins, have their own receptors and are able to induce cellular responses. The receptors of activins, e.g. Acr-IB and ActR-IIB are expressed on germ cells (195). The ectopic production of activin B from somatic cells of the fetal ovary in the absence of *Wnt4* and β--catenin may act directly on female germ cells and causes their deaths. Activins are known to stimulate cell signaling via SMAD2/3 pathway. Therefore, I propose to address this question by using biochemistry or immunological methods to detect whether SMAD2/3 pathway is activated in the female germ cells in the absence of *Wnt4* or β-catenin.

2. How does β-catenin suppress the expression of *Inhbb*?

In Chapter 3 and 4, I have demonstrated that β-catenin suppressed the expression of *inhbb* in the fetal ovary. Several LEF/TCF consensus sequences are in the in the promoter region of *Inhbb*. Therefore, β-catenin may suppress the expression of *Inhbb* in the fetal ovary by binding to these response elements. An alternative mechanism is that β-catenin does not directly suppress the expression of *Inhbb*. A method to examine these possibilities is to perform chromatin immunoprecipitation assays (ChIP assay) using β-catenin antibody. If β-catenin regulates *Inhbb* by binding its promoter directly, ChIP assay will be able to pull down the promoter sequence of *Inhbb*.

3. What are other signaling pathways involved in the development of fetal ovary?

In the absence of RSPO1/WNT4/β-catenin signaling, female mice develop partial male characteristics but sex reversal does not occur. These observations suggest that RSPO1/WNT4/β-
catenin signaling only regulates the ovarian development in mice partially and there may be other factors involved.

One of the candidate signaling pathways to regulate the ovarian development is Hedgehog (Hh) pathway. The components of Hh signaling pathway are known to be present in the fetal ovary. In addition, fetal Leydig cells appear when the Hh pathway is activated in the fetal ovary (215). This result indicates that the inhibition of Hh pathway during ovarian-organogenesis is required (215). Furthermore, the interaction between Hh pathway and WNT pathway has been demonstrated in mice and other organisms (216-218). For example, the cantonal WNT signaling activates a downstream element of Hh, Gli3 to inhibit the ventral cell fate program in chicken (218). Therefore, it will be interesting to further study the relationship between WNT and Hh pathways in the ovarian development.

In addition to the Hh pathway, FOXL2 is the other candidate that plays a different role from RSPO1/WNT4/β-catenin signaling in the ovarian development. FOXL2 belongs to a forkhead transcription factor family and expresses specifically in the ovary at the time of sex determination (22). Null mutation in Foxl2 resulted in the granulosa cell defects and premature ovarian failure. However, sex reversal did not occur in the Foxl2 KO mice (22, 41, 42, 68, 69, 71). The ovarian phenotypes in the Rspo1, Wnt4, KO or β-catenin cKO were different from those in the Foxl2 KO indicated that RSPO1/WNT4/β-catenin signaling pathway and Foxl2 may act independently in regulating the ovary development. However, when both Foxl2 and Wnt4 were inactivated in the mouse ovary, female-to-male sex reversal and irregular germ cell development occurred (72). In addition, Sox9, a factor that promotes the testis program, was transiently expressed in Wnt4−/− at 11.5 dpc and upregulated perinatally in Foxl2−/− (73-75). Theses results suggest that WNT4 and FOXL2 may operate synergistically to suppress the testis pathway, i.e. by inhibiting Sox9 expression, in the ovary and to establish the proper development of fetal mouse ovary. In addition, activation of β-catenin in the fetal gonads causes the upregulation of FOXL2 in both ovary and testis, implicating an intimate connection exists between β-catenin and FOXL2(172). FOXL2, as a transcription factor, can be one of the downstream target genes of β-catenin. Thus, exploring the molecular connection between β-catenin and FOXL2 is demanded to solve the puzzle of ovary development.
BIBLIOGRAPHY

17. E. Pailhoux et al., *Dev Dyn* 224, 39 (May, 2002).
86. E. A. Bosman et al., *Development* **133**, 3399 (Sep, 2006).
200. R. Widelitz, Growth Factors 23, 111 (Jun, 2005).
201. T. Kamata et al., Biochim Biophys Acta 1676, 51 (Jan 5, 2004).
203. N. Harada et al., EMBO J 18, 5931 (Nov 1, 1999).
209. O. Wirths et al., Lab Invest 83, 429 (Mar, 2003).
211. M. Uhmhauer et al., Embo J 19, 4944 (Sep 15, 2000).
213. T. Miyakoshi et al., Endocr Pathol 19, 261 (Winter, 2008).
214. A. Biason-Lauber et al., Hum Reprod 22, 224 (Jan, 2007).
APPENDIX A: The PCR reactions

*Ctnnb1 WT Genotyping*

Cycle 1: 94°C for 2 minutes

Cycle 2: 94°C for 1 minute

Cycle 3: 55°C for 1 minute

Cycle 4: 72°C for 45 seconds

Repeat cycles 2-4 for 35 more times

Cycle 5: 72°C for 2 minutes

Product size: Wild type = 221 bp; Floxed allele = 324 bp

*Ctnnb1 null allele Genotyping*

Cycle 1: 94°C for 2 minutes

Cycle 2: 94°C for 1 minute

Cycle 3: 55°C for 1 minute

Cycle 4: 72°C for 45 seconds

Repeat cycles 2-4 for 35 more times

Cycle 5: 72°C for 2 minutes

Product size: KO allele = 631 bp

*SF/1cre Genotyping*

Cycle 1: 94°C for 2 minutes

Cycle 2: 94°C for 45 seconds

Cycle 3: 55°C for 45 seconds
Cycle 4: 72 °C for 1 minute
Repeat cycles 2-4 for 34 more times
Cycle 5: 72 °C for 2 minutes

Product size: Cre positive = 408 bp; Cre negative = no band

**Sry Genotyping (to sex embryos)**

Cycle 1: 94°C for 7 minutes
Cycle 2: 92 °C for 1 minutes
Cycle 3: 55°C for 1 minutes
Cycle 4: 72 °C for 45 seconds
Repeat cycles 2-4 for 34 more times
Cycle 5: 72 °C for 5 minutes

Product size: Female = 300 bp; XY = 300 bp and 280 bp.

**Inhbb WT allele Genotyping**

Cycle 1: 95°C for 5 minutes
Cycle 2: 95 °C for 1 minutes
Cycle 3: 60°C for 1 minutes
Cycle 4: 72 °C for 1 minutes
Repeat cycles 2-4 for 34 more times
Cycle 5: 72 °C for 7 minutes

Product size: 138 bp fragment will appear in both wild type and heterozygotes.

**Inhbb null allele Genotyping**
Cycle 1: 94°C for 3 minutes
Cycle 2: 94 °C for 20 seconds
Cycle 3: 54.6°C for 30 seconds
Cycle 4: 72 °C for 21 minutes
Repeat cycles 2-4 for 34 more times
Cycle 5: 72 °C for 2 minutes
Product size: 1300 bp fragment will appear in both heterozygotes and knockouts.

**Wnt4 Genotyping**
Cycle 1: 94°C for 7 minutes
Cycle 2: 92 °C for 1 minutes
Cycle 3: 60°C for 1 minutes
Cycle 4: 72 °C for 45 seconds
Repeat cycles 2-4 for 34 more times
Cycle 5: 72 °C for 5 minutes
Product size: WT allele = 524 bp; Null allele= 300 bp.

**Ctnnb1**<sup>f(ex3)</sup> Genotyping
Cycle 1: 94°C for 1 minutes
Cycle 2: 94 °C for 30 seconds
Cycle 3: 60°C for 1 minutes
Cycle 4: 72 °C for 1 minute
Repeat cycles 2-4 for 34 more times

Cycle 5: 72 °C for 7 minutes

Product size: Wild type = 900 bp; Floxed allele = 1.4 kb

**RT-PCR reaction for Fzd and 36 B4**
Cycle1: 94°C for 3 minutes
Cycle 2: 94 °C for 1 minutes
Cycle 3: 55°C for 1 minutes
Cycle 4: 72 °C for 2 minutes
Repeat cycles 2-4 for 32 more times
Cycle 5: 72 °C for 5 minutes

**Quantitative PCR reaction:**
Cycle1: 95°C for 3 minutes
Cycle 2: 95 °C for 10 seconds
Cycle 3: 55°C for 30 seconds
Repeat cycles 2-3 for 49 more times
APPENDIX B

1. Study of *Amh* and *Inhbb* double knockout male mice

At the fetal stage, meiosis only occurs in the female germ cells but not in the male germ cells. However, if culturing the XX gonad with the fetal testis, the meiosis of female germ cells was prevented from reaching the diplotene stage. Thus, it was suggested that the meiosis-preventing substance in the fetal testis prevented the meiosis of male germ cells (102-108). Only recently, it is unveiled that the initiation of meiosis is regulated by the retinoic acid signaling (116). At the time I initiated this project, the role of retinoic acid signaling on germ cell meiosis was still unknown. The original objective of this study was to indentify the potential meiosis-preventing substance that prevented male germ cells from entering meiosis. activin B and AMH are two of those potential substances. AMH is a testis-specific factor while inhibin b B (*Inhbb*), the gene that encodes Activin B, is maintained at a low level in the fetal ovary but is highly expressed in the fetal testis. Both *Amh* and *Inhbb* are specifically expressed in the Sertoli cells, which are known to be essential for male germ cell development. However, the meiosis of germ cells does not occur in the fetal testes of the *Amh* or *Inhbb* single knockout (KO) mice. Activin B and AMH both belong to the transforming growth factor β (TGFβ) superfamily, which has a common cell signaling pathway in regulating cell differentiation, proliferation, apoptosis, and homeostasis. I therefore hypothesized activin B and AMH compensate each other in inhibiting germ cell meiosis in testis. Thus, I generated *Amh* and *Inhbb* double KO mice to examine this possibility.

Materials and Methods

Animals

To obtain the *Amh* and *Inhbb* double KO mice (*Amh*+/−; *Inhbb*+/−), I first mated *Amh*+/− mice with *Inhbb*+/− mice to generate *Amh*+/−; *Inhbb*+/− double heterozygotes. *Amh*+/−; *Inhbb*+/− double heterozygotes were then mated to generate *Amh*+/−; *Inhbb*+/− double KO mice. The day when the vaginal plug was detected in the mated female was considered as 0.5 day post coitum (0.5 dpc).
**Immunohistochemistry**

Immunohistochemistry with TRA98 and SOX9 antibodies was used to detect germ cells and Sertoli cells in testis. Details on the procedure of immunohistochemistry were described in Chapters 2 and 3.

**Results and Discussion**

Although I did not observe the occurrence of meiosis of germ cells in fetal testis, I found that Amh<sup>−/−</sup>; Inhbb<sup>−/−</sup> male mice developed some interesting phenotypes. At 14.5 dpc, the shape of Amh<sup>−/−</sup>; Inhbb<sup>−/−</sup> testis was elongated comparing to control testis (Fig. B1 A & B, n=1). At birth, the shape of Amh<sup>−/−</sup>; Inhbb<sup>−/−</sup> testis was different from the control testis but its size was similar to the control testis (Fig. B2 A & B). Immunostaining with TRA98 and SOX9 antibodies showed that some of the TRA98-positive germ cells were located outside the testis cord at the edge of the testis (Fig. B2 C & D). Immunostaining with CYP17 antibodies revealed that the steroidogenic cells were present in the Amh<sup>−/−</sup>; Inhbb<sup>−/−</sup> testis (Fig. B2 E & F, n=2). In general, the morphology of Amh<sup>−/−</sup>; Inhbb<sup>−/−</sup> testis was resemble to the ovotesits (219) and the ovary-like characteristics tended to appear at the edge of testis. The ovarian-like structure appeared Amh<sup>−/−</sup>; Inhbb<sup>−/−</sup> testis suggesting Amh and Inhbb migh have functional role in controlling cell plasticity of the gonad. In addition, these ovary-like characteristics appeared in the testis with normal expression of SOX9, suggesting Amh and Inhbb might be involved in an antagonized ovarian pathway that is independent from Sox9 or Sry. Further experiments using ovary-specific markers are needed to confirm whether those phenotypes are ovary-specific.
Figures:

Fig. B1 The phenotypes of control and $Amh^{-/-}$; $Inhbb^{-/-}$ testis at 14.5 dpc. (A-B) whole mount light microscope images of control testis (A) and $Amh^{-/-}$; $Inhbb^{-/-}$ testis (B).
Fig. B2 The phenotypes of control and $Amh^{-/-}; Inhbb^{-/-}$ testis at birth. (A-B) whole mount light microscope images of control testis (A) and $Amh^{-/-}; Inhbb^{-/-}$ testis(B). (C-D) Immunostaining with TRA98 and SOX9 antibody on control (C) and $Amh^{-/-}; Inhbb^{-/-}$ testis (D), (E-F) Immunostaining with CYP17 antibody on control (E) and $Amh^{-/-}; Inhbb^{-/-}$ testis (F)
2. Assessment of the association between Smoothened activation mice and polycystic ovary syndrome

The excessive androgen production in the early fetal life is thought to be a cause of the polycystic ovary syndrome (PCOS). For example, studies on monkeys and sheep showed that exposing fetuses to a high level of androgen could result in the development of PCOS, including hyperandrogenism and insulin insensitivity (220, 221). However, uses of monkeys or sheep to study the etiology of PCOS are expensive. Thus, I explored the possibility of using mouse models to study whether the excessive androgen is associated with the PCOS. The ectopic androgen production was present in the Wnt4 knockout (KO) and β-catenin conditional knockout (cKO) female mice. However, both Wnt4 KO and β-catenin cKO mice would die on postnatal day one due to the dysgenesis of kidney and adrenal, respectively. Thus, the use of these mouse models to study this subject is impeded. Previous studies by the Yao’s lab showed that the ectopic androgen production was also present in other transgenic mouse model, the Smoothened (Smo) activation mouse line. The Smo gene encodes a transmembrane protein that is responsible for transducing the intracellular signaling pathway induced by the Hh ligands. When the Cre transgenic mouse line is crossed to the Smo-YFP mouse line, Cre recombinase removes the STOP sequence upstream of the SmoYFP transgene. The removal of the STOP sequence allows the transcription of a constitutively active form of mutated Drosophila Smoothened, which in turn activates the Hh pathway regardless of the presence or absence of the Hh ligands. Therefore, I conducted the experiments using the Smo activation mice in which Smo was constitutively activated in the anti-Müllerian hormone receptor-2 -positive cells (Amrh2) to study the association between the excessive androgen and the PCOS. The main advantage of using this model is that the mutants can survive after birth.

Materials and Methods:

Animals

Amhr2<sup>cre/+-</sup> mice, in which the Cre expression is driven by anti-Müllerian hormone receptor-2 (Amhr2), was generously provided by Dr. Richard Behringer. GT(Rosa)26Sor<sup>tm1(smo/YFP)Amc</sup>/J mice (Smo-YFP) were obtained from Jackson lab. Amhr2-Cre mice were mated with Smo-YFP mice to obtain Amhr2<sup>cre/+</sup>Smo-YFP mice (mutants) and
Amhr2+/+SmoYFP mice (controls). Mice were weighed weekly from 21 days of age to 14 weeks of age. The following procedures were concluded between 8 and 16 weeks of age.

**Vaginal smears**

The stage of estrous cyclicity was determined by microscopic analysis of the predominant cell type in the vaginal smears obtained daily from 8 weeks of age to 12 weeks.

**Body composition**

Body composition was examined by dual-emission X-ray absorptiometry (DEXA) instrument (HOLOGIC Discovery-A Bone Densitometer) at 12 weeks of age. Mice were anesthetized using Ketamine/medetomidine (75 mg/kg, intraperitoneal injection) before scanning. Global body fat (FAT), lean body mass (LBM), global mass, bone mineral content (BMC), and bone mineral density (BMD) were measured.

**Glucose tolerance test (GTT)**

This test was performed at 15-16 weeks of age. Mice were fasted overnight (14 to 18 hours) with full access to water. Mice were intraperitoneally injected with sterile glucose in distilled water (75 mg/ml) at a dose of 1.5 mg per gram body weight. A drop of blood was collected from tail at 0, 15, 30, 60, and 120 minutes after glucose injection. Blood glucose level was measured using the one-touch basic glucometer (FreeStyle FREEDOM).

**Insulin sensitivity test**

At 17-18 weeks of age, mice were subjected to insulin sensitivity tests. Mice were intraperitoneally injected with regular human insulin (0.75 units per kilogram body weight) and blood glucose level was measured before insulin injection and at 15, 30, 60, and 90 minutes after injection. Blood glucose level was measured using the one-touch basic glucometer (FreeStyle FREEDOM).

**Statistical analysis**

General linear model with repeated measures was used to compare mean body composition measures and mean blood glucose level between mutant and control groups.
Tukey’s tests for pirewise comparison at each time point were performed if the overall pattern between the two groups was significant (i.e., a $p \leq 0.05$).

Results

The results were from two litters of mice. The first group had two control and three $Amhr2^{cre/+}Smo$-YFP mice and the second group had four control and six $Amhr2^{cre/+}Smo$-YFP mice.

The estrous cyclicity of the Smoothened activation female mice was disrupted

All control females ($n=6$) had a normal 4 or 5-day estrous cycle (Fig. B3, only three of the representative mice were present) while seven out of the nine $Amhr2^{cre/+}Smo$-YFP females had an irregular estrous cycle (Fig. B3, three of the seven representative mice were present). The average number of estrous cycles during a 21-day period was 4.8±0.3 in the control mice and was 2.1±0.9 in the $Amhr2^{cre/+}Smo$-YFP females.

Body weight of the Smoothened activation female mice was increased

The Smoothened activation female mice ($Amhr2^{cre/+}Smo$-YFP) started to show a significant increase in body weight ($p<0.05$) comparing to the controls at 10 weeks of age (Fig. B4).

Smoothened activation female mice did not have more body fat

There was no significant difference in the global body fat (Fig. B5) between control and $Amhr2^{cre/+}Smo$-YFP mice. However, further investigation revealed a significant interaction between litter and genotype on global body fat (Fig. B6). In the first group (two controls and three $Amhr2^{cre/+}Smo$-YFP mice) the mean global body fat was significantly higher in the $Amhr2^{cre/+}Smo$-YFP female mice than in the controls ($p=0.038$). No significant difference was found in the second group (Fig. B6). The different results found between the two litters suggested that there was variation in global body fat both among litters and individuals. The global body mass ($p=0.005$) and the lean body mass ($p=0.006$) were significantly different between control and the $Amhr2^{cre/+}Smo$-YFP female mice (Fig. B5), suggesting that the increase in body weight in the $Amhr2^{cre/+}Smo$-YFP female mice was resulted from the increase in lean
body mass. The data also implicated that Amhr2cre/+ Smo-YFP females did not have obesity. However, further evaluation for obesity in mice using standard method is needed.

**Smoothened activation female mice did not develop glucose intolerance**

There was no difference in the temporal pattern of blood glucose level between control and Amhr2cre/+ Smo-YFP groups (p=0.365), suggesting that activation of Smo in female mice did not cause glucose intolerance (Fig. B7 A).

**Smoothened activation female mice did not develop insulin insensitivity**

Because the first litter did not survive, I only performed the insulin insensitivity tests on the second litter, which had four control and six Amhr2cre/+ Smo-YFP mice. No difference between the two groups was observed (p=0.949), suggesting that activation of Smo in female mice did not cause insulin insensitivity (Fig. B7 B).

In summary, the Amhr2cre/+ Smo-YFP female mice did not develop metabolism problems. The findings did not support that the excessive androgen productions in the early fetal life was associated with the PCOS as metabolism problems, such as diabetes and obesity, were the common outcomes in the PCOS patients. However, the results from this preliminary study are not consistent, suggesting further investigations are demanded. The observations on the variation of global body fat between the two litters further suggest that the individual mice might have a various degree of phenotypes. Because both human PCOS patients and PCOS animal models indicate that hyperandrogenism is the major marker of PCOS (222), further experiment to evaluate androgen (e.g., testosterone) level and its association with the phenotypes of Amhr2cre/+ Smo-YFP mice is promising.
Figures

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Day 1 3 5 7 9 11 13 15 17 19 21

Fig. B3 Estrous cycle patterns from 8 weeks of age to 12 weeks in three represent mice from control and *Amhr2cre/+Smo-YFP*. D, Diestrus; E, estrus, M, metestrus; P, proestrus.
Fig. B4 Growth curves of control mice and smo activated female (Amhr2cre/+Smo-YFP) mice. The mean weight starts to show statistically significant difference (i.e., p<0.05) at week 10. The asterisk represents statistical significance.
Fig. B5 The preliminary results of body composition analysis using DEXA machine in the control mice and smo activated female (*Amhr2cre/+Smo-YFP*) mice. FAT= globe body fat, LEAN=globe lean mass, MASS= global mass. The asterisk represents statistical significance.
Fig. B6 The preliminary results of global body fat using DEXA machine in two different group. The asterisk represents statistical significance.
Fig. B7 The glucose tolerance test (GTT) and Insulin sensitivity tests (IST) in the control mice and smo activated female (Amhr2cre/+Smo-YFP) mice. The overall temporal changes in GTT (A) and IST (B) are present.
CURRICULUM VITAE

Chia-Feng Liu

1807 Valley Rd Champaign, IL 61820,USA
Tel: 1-217-898-6857
E-mail: cliu26@illinois.edu

EDUCATION

2010 Ph.D University of Illinois at Urbana-Champaign
Department of Veterinary Biosciences
Advisor: Dr. Humphrey H-C Yao
Dissertation Title: The role of beta-catenin in the development of fetal ovary and female germ cells

2001 M.S National Taiwan University.
Institute of Fisher Science _Developmental Biology
Master’s Thesis: Identification of somite-specific cis-acting elements of zebrafish (Danio rerio) myf-5 gene by using transgenic fish.

1999 B.S Fu-Jen Catholic University, Taipei, Taiwan
Major in Biology

HONORS AND AWARDS

2009 NIH National Graduate Student Research Festival. Invited by NIH to attend the pre-doctoral conference hold in the NIH.

2009 SDB Student/Postdoc Best Poster Award. Awarded by the Society of Developmental Biology, 2009. 68th Annual Meeting, San Francisco, California, USA.

2009 SDB Student/Postdoc Travel Award. Awarded by the Society of Developmental Biology.


2007-2009  Graduate Student Travel Award. Awarded by the University of Illinois at Urbana-Champaign.

2001  The Dean’s List Award of Graduate College for Outstanding Academic Achievement. Awarded by National Taiwan University, Taiwan.

2001  The Poster Thesis Award at Graduation. Awarded by the Institute of Fisheries, National Taiwan University, Taiwan.

2001  The Best Poster Thesis Award of the 16th Joint Annual Conference of Biomedical Sciences, 2001. The 16th Joint Annual Conference of Biomedical Sciences, Taipei Taiwan. Awarded by the Taiwan Society for Biochemistry and Molecular Biology.

RESEARCH EXPERIENCE

2004-2005  Research Assistant, National Health Research Institute.
Studying the antibiotic resistance mechanism of Klebsilla pneumoniae. Advisor: Dr. Leung-kei Siu, Division of Clinical Research.

1997-1998  Summer Internship, National Taiwan University.
Participated on screening and cloning of c-ski gene of tilapia. Advisor: Dr. Huai-Jen Tsai, Institute of Fisheries Science.

1996.6-9  Summer Internship, National Yang-Ming University 6/96-9/96
Participated in screening microbial enzymes, d-Aminoacylase, for industrial utility. Advisor: Ying-Chieh Tsai, Institute of Biochemistry.

TEACHING EXPERIENCE

2009.5  Teaching Assistant- Frontiers in Reproduction Lab, Marine Biological Laboratory.
Prepared Lab material and demonstrated experimental procedures to the class.

2001-2004  Teaching Assistant – Gene Technology Lab, Biochemistry Lab and Cell Biology Lab Fu-Jen Catholic University, 8/01-7/04
Gave lectures on methodology of molecular biology, biochemistry and cell biology analysis, Prepared Lab materials and demonstrated experimental procedures to the class. Assisted the instructor with preparation of lecture materials and graded assignments.

2001-2004  Teaching Assistant – Biochemistry Lab, Fu-Jen Catholic University, 8/01-7/04
Gave lecture on experimental methods of biochemistry, including protein extraction, Enzyme-Linked Immunosorbent Assay (ELISA), protein gel filtration,
affinity chromatography, ion exchange chromatography of amino acids, isolation of RNA and electrophoresis of RNA, thin layer chromatography, measurement of intracellular free Ca\(^{2+}\) concentration, and tutored students. Prepared Lab materials and demonstrated experimental procedures to the class. Assisted the instructor with preparation of lecture materials and graded assignments. Dr. Wan-Fang Tzeng, Department of Life Science.

2001-2004  **Teaching Assistant** – Cell Biology Lab, Fu-Jen Catholic University, 8/01-7/04
Gave lecture on experimental methods of cell biology, including cell culture techniques, flow cytometry analysis, MTT test, organelle isolation. Prepared Lab materials and demonstrated experimental procedures to the class. Assisted the instructor with preparation of lecture materials and graded assignments. Dr. Wan-Fang Tzeng, Department of Life Science.

**PUBLICATION**

**Research Articles**

**Liu C-F**, Parker, K, Yao HH. Wnt4/β-catenin Pathway Maintains Female Germ Cell Survival by Inhibiting Activin bB in the Mouse Fetal Ovary.


Chen YH, Lee HC, **Liu C-F**, Lin CY, and Tsai HJ. Novel Regulatory Sequence -82/-62 Functions as a Key Element to Drive the Somite-Specificity of Zebrafish *myf*-5. *Dev. Dyn.* 2003 Sep;228(1):41-50


**Book Chapters**

Abstracts


