博士論文 (要約)

Studies on Partial Masculinization in Mouse Embryonic Ovaries Grafted into Male Nude Mice

(雄ヌードマウスに移植したマウス胎子卵巣の雄性化機構に関する研究)

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General Introduction

In mammals, testis and ovary are critical organs producing gametes, which give life to the next generation by producing sperm or oocytes. Mammalian sex is determined based on sex chromosome constitution (i.e., XY or XX) at the time of fertilization. In most mammals, including humans and mice, both XY and XX embryos develop equally in a non-sexually dimorphic fashion until the early organogenic stage, leading to the formation of long and narrow gonadal primordia along the mesonephric region of the posterior trunk (Fig. 1A). Such bipotential gonads develop into either testes or ovaries in the presence or absence of *Sry, Sex-determining region Y gene*, at the critical time window during 11.0 days post coitum (11.0 dpc) –11.5 dpc in mice (see reviews by Kashimada and Koopman 2010; Harikae et al. 2013a; Larney et al. 2014; Fig. 1B). After gonadal sex determination, the differentiating gonadal somatic cells produce various sex-dimorphic signaling factors for the maintenance of each sex of the supporting cells and simultaneously secrete testis- or ovary-specific hormones that affect the sexually dimorphic development of the intra- and extra-reproductive organs during the late fetal and peri- and postnatal stages. Such sexually dimorphic hormonal secretion from the gonads results in the sexual maturation of the adult male or female.

Vertebrate sex determination is called “one tissue, two fate” (Brennan and Capel, 2004) because one of two organs, testis or ovary, develops from a bipotential gonad. Therefore, cell linages consisting of testis or ovary, such as germ cells, supporting cells, steroidogenic cells, and so on, have counterparts originated from the same precursors in undifferentiated gonads. Briefly, both mammalian testes and ovaries consist of the same cell linages, germ cells (i.e., spermatogenic cells and oocytes) and supporting cells (i.e., Sertoli cells and granulosa cells) inside a tubular or follicular structure, which is surrounded by steroidogenic cells (i.e., Leydig cells and internal theca cells) and myoid cells in the interstitial regions (Fig. 1C). In Sertoli cell differentiation of mouse XY gonads, SRY is transiently activated in only supporting cells and

upregulates SOX9 (SRY-related HMG-box 9), leading to male-specific gene expressions (e.g., *Sox8*, *Fgf9* [*fibroblast growth factor 9*], *Gdnf* [*glial cell-line derived neurotrophic factor*], and *Amh* [*anti-Müllerian hormone*]). Differentiating pre-Sertoli cells play a central role in the male sex determination of the other cell lineages, such as germ cells, interstitial steroidogenic cells, and vascular patterns (Svingen and Koopman, 2013). Testis cords, tubular structures of germ and Sertoli cells packed within basal lamina, are formed in XY gonads at fetal stage (Harikae et al., 2013a). After birth, the testis cords develop into seminiferous tubules where spermatogenesis proceeds continuously. In the absence of SRY, most XX supporting cells lose SRY-dependent SOX9 inducibility (SDSI; Hiramatsu et al., 2009; Harikae et al., 2013b) and express *Foxl2* (*forkhead box L2*), becoming pre-granulosa cells which cause female sex differentiation in the other cell lineages. However, a subpopulation of the pre-granulosa cells near the mesonephric tissue maintains SDSI throughout fetal and early postnatal stages, even after they come to be FOXL2-positive at fetal stage. In XX gonads, ovigerous cords consisting of germ cell cysts and pre-granulosa cells are formed at fetal stage (Pepling, 2006; Hummitzsch et al., 2013; Suzuki et al., 2015). After birth, these cysts break down into each primordial follicle consisting of an oocyte and a single layer of flat pre-granulosa cells. Some of these primordial follicles develop into AMH-positive primary, secondary, or antral follicles in the medullary region of ovaries, as initial round of folliculogenesis (Mork et al., 2012; Shinomura et al., 2014; Suzuki et al., 2015). The subpopulation of the pre-granulosa cells with SDSI contributes to the initial round of folliculogenesis by secondary follicle stage (Harikae et al., 2013b). In contrast, other dormant primordial follicles in the cortex region are recruited and activated in the cyclical and selective manners, which subsequently results in the consecutive waves of the cyclical follicular activation. Little is known about the biological significance of first wave of

folliculogenesis, partly because there are only a few models to analyze first wave follicles (Mork et al., 2012; Shinomura et al., 2014; Zheng et al., 2014).

Another feature of mammalian sex determining process is that the sex determination/differentiation of the fetal gonads proceeds inside the mother's womb (i.e., in an estrogen-rich environment) through the placenta. Such an estrogen-dominant environment may lead to the low sensitivity to estrogens in most parts of the sex-determination process, at least during the fetal stages of mammals. In fact, steroidogenic cell differentiation and hormone production occurred after birth in XX gonads (Honda et al., 2007; Young et al., 2010; Liu et al., 2015; Miyabayashi et al., 2015). And *estrogen receptor* (*Esr*) *1/2* double-null females showed their phenotype of ovotestis formation only after birth (Couse et al., 1999; Dupont et al., 2003). In contrast, male hormones, such as AMHs and androgens, produced in embryonic testes have the dominant regulation in Müllerian duct degeneration and masculinization of brain and external reproductive organ in fetal stage (Behringer et al., 1994; Geissler et al., 1994; Hu et al., 2002; Sato et al., 2004; O'Shaughnessy et al., 2011).

DSD (disorders of sex-development) is defined as "congenital conditions in which the development of chromosomal, gonadal, or anatomical sex is atypical" (Hughes et al., 2006; Bashamboo and McElreavery, 2015). This definition includes errors of primary sex determination, such as 46,XY complete or partial gonadal dysgenesis (CGD, PGD), 46,XX testicular DSD, and 46,XX ovotesticular DSD. Some cases of 46,XX testicular/ovotesticular DSD could be explained by the translocation of *SRY* to the X chromosome or an autosome, overexpression of pro-tests genes including *SOX* family genes, or failure of pro-ovarian/anti-testis genes (Grinspon and Rey, 2016), but other cases couldn't be explained by the mechanism related with such genes. Some cases of the virilization of the XX fetus were the result of excessive androgen action during intrauterine development, resulting from virilizing tumors

and congenital adrenal hyperplasia in humans (Grinspon and Rey, 2016). Such fetal disorders involving excess androgen permit proper ovarian differentiation, but in some cases, lead to prevalent hyperandrogenic infertility of polycystic ovary syndrome accompanied by excess AMH (Abbotte and Bacha, 2013). Moreover, freemartin syndrome causes infertility in a female cattle twin born with a male twin (Marcum, 1974; Padula, 2005). Since the female bovine fetus shares a blood supply with the male fetus, some circulating factors derived from the male twin (e.g., testosterone and AMH) may cause masculinization of the genital organs of the female twin, including testis-like structures with SOX9-positive Sertoli-like cells in some severe cases (Harikae et al., 2012), but this molecular mechanism remains unknown. Similar to such freemartin ovaries, the fetal mouse ovarian grafts under the kidney capsule of adult male mice undergo a partial sex-reversal showing the ectopic formation of the testis cord-like structures, together with the follicular degeneration and subsequent ectopic appearance of SOX9-positive Sertoli-like cells (Taketo et al., 1984; Taketo and Merchant-Larios, 1986; Morais da Silva et al., 1996; Harikae et al., 2013b; Fig. 2). These findings suggest that a switch from a maternal-to-paternal environment induces a partial masculinization of the fetal ovaries even in normal wild-type genotype. However, the contribution of paternal environment of host male mice to such masculinization of fetal ovaries hasn't been examined in detail. With the ovarian grafting experiment into the male nude mice, Harikae (2013) showed the transition of sex determination-related genes in such a partial masculinization of granulosa cells in the first wave of folliculogenesis. However, among these altered sex determination-related genes, it is still unclear what genes contribute to the follicular degeneration, tubular structure formation, and ectopic SOX9-positive Sertoli cell-like cell appearance in the partial masculinization in the ovarian grafts under the paternal environment.

In this study, I tried to reveal the mechanism of the differentiation and masculinization of XX supporting cells by analysis of XO mouse ovaries and mouse ovarian grafts into male nude mice. In chapter 1, I did histological analysis on XO postnatal ovaries to reveal the granulosa cell differentiation in the first wave of folliculogenesis. In chapter 2, in order to reveal to what extent the grafted ovaries re-acquire the sexual bipotency, I induced ectopic SRY in grafted ovaries in an *Sry*-inducible system and examined the upregulated genes. In chapter 3, in order to examine the contribution of male host environment to this masculinization including the ectopic appearance of SOX9-positive Sertoli-like cells, we transplanted fetal mouse ovaries into healthy male, healthy female, or castrated male host mice with or without silicon tubes containing testosterone. In chapter 4, I examined the temporal changes of Sertoli cell-specific transcription/nuclear factors in grafted fetal ovaries during the partial masculinizing process. I also identified the spatiotemporal expression patterns of *Sox8* in the ovarian grafts and examined the roles of donor-derived *Sox8* and *Amh* action in such masculinizing processes including follicle degeneration and ectopic appearance of SOX9-positive Sertoli cell-like cells.

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Figure 1 of General Introduction: Gonadal sex differentiation and its principle structure in mammals

(A, B) Schematic representation showing mouse embryo with the genital ridges extended along the anteroposterior axis of the posterior trunk (A), and male and female gonads before and after sex determination at 10.5 dpc and 13.5 dpc (B). The dissecting microscopic images of the testis and ovary at 13.5 dpc are also shown in the right-hand side (note testis cords [in future seminiferous tubules] in the testis). **(C)** Schematic representation and HE-stained images showing the seminiferous tubule and ovarian follicle at the postnatal and adult stages. In both testis and ovary, gonadal supporting cells (i.e., Sertoli and granulosa cells) and germ cells are tightly packed within the basal lamina layer, which forms the seminiferous epithelium or ovarian follicle. Steroidogenic cells (i.e., Leydig cells and theca cells) and myoid cells are located in the interstitium.

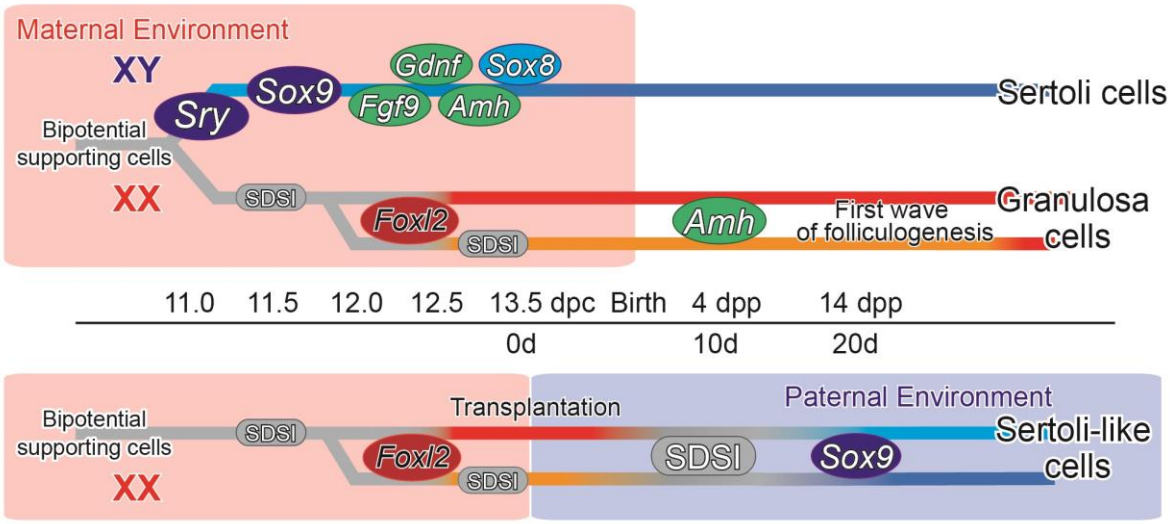

Fig. 2 of General Introduction

Figure 2 of General Introduction: Sex differentiation and partial masculinization in mouse supporting cells

In sex determination of mammalian XY supporting cells, during 11.0–12.0 dpc, SRY promotes SOX9 expression in a male-specific manner, leading to pre-Sertoli cell differentiation. In pre-Sertoli cells, SOX9 induces various testis-specific factors, such as *Fgf9*, *Gdnf*, *Amh*, and *Sox8*, leading to Sertoli cell differentiation and testis formation. In XX supporting cell differentiation, pre-granulosa cells maintain SDSI until 11.5 dpc. After that stage, a subpopulation of most pre-granulosa cells rapidly loses this ability by 12.0 dpc and comes to express *Foxl2*. However, another subpopulation of the pre-granulosa cells near the mesonephric tissue maintains SDSI throughout fetal and early postnatal stages, even after they come to be FOXL2-positive at fetal stage. After birth, both FOXL2-positive subpopulations contribute to the initial round of folliculogenesis together with AMH expression, but SDSI is lost in the later population by the secondary follicle stage.

Ovarian transplants into male mice show a partial masculinization. Granulosa cells in grafted ovaries re-acquire SDSI by day 10 post-transplantation. In the masculinizing process, *Sox9* expression is detected in the grafted ovaries by day 20 post-transplantation.

Chapter 1

Defects in the first wave of folliculogenesis of neonatal ovaries in XO female mice

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Chapter 2

Re-acquisition of sexual bipotency

in fetal mouse ovarian grafts into male nude mice

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Chapter 3

Partial masculinization of fetal mouse ovarian grafts caused by testosterone derived from male host mice

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Chapter 4

Potential roles of *Sox8* and *Amh* for partial masculinization in ovarian grafts into male nude mice

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General Discussion

In sex determination of mammalian XY supporting cells, SRY promotes SOX9 expression during 11.0–12.0 dpc, leading to pre-Sertoli cell differentiation. Around the same stage, expressions of potential SRY target genes, such as *Zfp532*, *Lmo4*, and *Mamld1*, are upregulated in a male-specific manner (chapter 2). In pre-Sertoli cells, SOX9 induces various testis-specific factors, such as *Fgf9*, *Gdnf*, and *Amh*, leading to Sertoli cell differentiation and testis formation. *Egr1/2*, *Nr4a1/2*, *Zc3h12c*, and other transcription factors also show Sertoli cell-specific upregulation during 12.5 dpc (chpter 4).

In XX supporting cell differentiation, pre-granulosa cells are divided into two subpopulations. Almost all pre-granulosa cells maintain SDSI until 11.5 dpc (Hiramatsu et al., 2009; Harikae et al., 2013b). After that stage, a first subpopulation of most pre-granulosa cells rapidly loses this ability by 12.0 dpc and comes to express *Foxl2*. However, a second subpopulation of the pre-granulosa cells near the mesonephric tissue maintains SDSI throughout fetal and early postnatal stages, after they come to be FOXL2-positive at fetal stage (Harikae et al., 2013b). After birth, both FOXL2-positive subpopulations contribute to the initial round of folliculogenesis together with AMH expression, but SDSI in the second subpopulation is lost by the secondary follicle stage. In chapter 1, by means of XO mice, I show the SDSI is maintained in first wave granulosa cells in an oocyte-independent manner but AMH expression may be regulated in oocyte growth-dependent mechanism.

Ovarian transplants into male mice show a partial masculinization. Granulosa cells in grafted ovaries re-acquire sexual bipotency, not only SDSI but also SRY-dependent inducibility of *Fgf9*, *Gdnf*, and potential SRY target genes, by day 10 post-transplantation (chapter 2). Harikae et al. (2013b) showed a considerable number of ovarian granulosa cells in not only the ovarian medullary region but also throughout the ovarian parenchyma gradually re-acquire the SDSI on days 7 and 10 post-transplantation. Therefore, these data indicate that during the partial

masculinization, almost all granulosa cells in the first and second subpopulations can require or maintain the sexual bipotency evaluated by monitoring SDSI and the inducibilitiy of other testicular genes.

I show that *Sox8* and *Amh* are positively involved in the survival of follicles in the ovarian grafts in the male mice in chapter 4. Throughout the ovarian parenchyma, the both signals of *Sox8* and *Amh* are detected in follicles in wild-type grafted ovaries and degenerating follicle are observed in *Sox8*-null and *Amh*-null transplants. Therefore, during the partial masculinizing process, *Sox8* and *Amh* modulate follicular degeneration and tubular structure formation in granulosa cells in both first and second subpopulations.

In chapter 3, I reveal that testosterone derived from male host may be involved in the appearance of SOX9-positive Sertoli cell-like cells in the ovarian explants grafted into male host mice. However, the number of SOX9-positive cells is relatively small compared with SDSI-positive granulosa cells (Harikae et al., 2013b), and they are detected in the medullary region in grafted ovaries into healthy male and castrated male mice with testosterone. So, these results indicate that although granulosa cell in the first subpopulation in the whole ovarian parenchyma could re-acquire sexual bipotency during the partial masculinization but they don't become SOX9-positive cell, sexually bipotential granulosa cells in the second subpopulation in the medullary region become SOX9-positive Sertoli cell-like cells. These sexually bipotential granulosa cells may be involved in the ovotestis formation in the ovarian centromedullary region in some transgenic mouse lines (Couse et al., 1999; Dupont et al., 2003, Schmidt et al., 2004; Uda et al., 2004; Ottolenghi et al., 2007, Chassot et al., 2008; Maatouk et al., 2013). In addition, upregulated Sertoli cell-specific transcription/nuclear factors, including *Egr1/2*, *Nr4a1/2*, and *Zc3h12c*, in the ovarian transplants and other SOX/TGF-beta family genes may

be involved in the follicular degeneration and tubular structure formation in the two subpopulations, or the upregulation of *Sox9* in the second subpopulation.

Sex determination mechanism has high variety in the animal kingdom, and in fact, *Sry* is found only in mammals, with the exception of monotremes, the Ryukyu spiny rat, etc. (Wallis et al., 2007; Kuroiwa et al., 2010; Cortez et al., 2014; Graves, 2016). In mammalian testis determination, SRY on Y chromosome activates SOX9 in supporting cells, leading to Sertoli cell differentiation at fetal stage in mother's womb, estrogen-rich environment. In contrast, many other vertebrates without SRY determine their sex at fetal stages in their eggs, self-produced hormone-rich environment. Gonadal differentiation to testis occurs in *Ar*-null male mice, even though they show spermatogenic arrest and developmental failure of adult Leydig cells (O'Shaughnessy et al., 2002; De Gendt et al., 2004). Therefore, the mammalian-specific SRY-dependent and male hormone-independent testis determination mechanism may have been acquired evolutionally to enable testis formation in estrogen-rich maternal environment. Among other vertebrates including frog and chicken, hormone sex determination is conserved mechanism and many cases of hormone-dependent sex reversal are reported (Villalpando and Merchant-Larios, 1990; Crews et al., 1991; Elbrecht and Smith, 1992; Shibata et al., 2002; Ohtani et al., 2003; Leet et al, 2011; Piprek et al., 2012). Therefore, the SRY-independent and testosterone/paternal environment-dependent partial masculinization in the grafted mouse ovaries may use male hormone-dependent testis determination pathways conserved among other vertebrates.

The present study indicated that oocyte growth induces AMH expression in granulosa cell differentiation (chapter 1) and AMH serves as a survival factor for follicle growth (Visser et al., 2007; chapter 4), possibly in a positive-feedback manner. This is consistent with the findings that the ovarian mouse grafts show AMH-positive follicular degeneration, oocyte loss, and

subsequent tubular structure formation with loss of AMH expression in the partial masculinization (Harikae et al., 2013b; chapter 3, 4). In medaka, germ cells are required for the ovarian formation and granulosa cell maintenance, and germ cell-deficient medaka develops as male irrespective of genetic sex (Kurokawa et al., 2007; Nishimura and Tanaka, 2014). Therefore, it is indicated that the germ cell-dependent sex determination mechanism of other vertebrates is potentially conserved even in mammals, and oocyte loss contributes to tubular structure formation and Sertoli cell-like cell appearance in the partial masculinization of grafted mouse ovaries into male mice.

In addition, the partial masculinization in mice, as well as testis determination in some other vertebrates, shows the onsets of *Amh* expression prior to *Sox9* expression (Westerm et al., 1999; Oréal et al., 2002; Yao and Capel, 2005; Klüver et al., 2007), but in contrast, SOX9 upregulates *Sox8* and *Amh* in mammalian testis determination (De Santa Barbara et al., 1998; Schepers et al., 2003). Therefore, the partial masculinization including the follicular degeneration and tubular structure formation relative with *Sox8* and *Amh* and the subsequent testosterone-dependent ectopic appearance of SOX9-positive Sertoli cell-like cells in mouse grafted ovaries might use the common and conserved molecular pathway among the male sex determination process of other vertebrates. Further studies about other *Sox* genes, TGF-β family members, and Sertoli cell-specific genes encoding transcription/nuclear factors upregulated in ovarian grafts, including *Egr1/2, Nr4a1/2*, and *Zc3h12c* (Table 4-4), will reveal the critical gene for the partial masculinization including the ectopic appearance of SOX9-positive Sertoli cells in mammals, and the genes playing a role in sex determination in other vertebrates.

In humans, fetal disorders involving excess androgen lead to prevalent hyperandrogenic infertility of polycystic ovary syndrome in some cases but their mechanism hasn't understood completely (Abbotte and Bacha, 2013). Also in cattle, "freemartin" syndrome female cattle

born twin to a male shows infertility and the disease causes a severe impact on the livestock industry. The present studies give the knowledge about the maintenance of sexual bipotency and the differentiation in granulosa cell in first wave of folliculogensis in XO mice, and reveal the molecular mechanisms of the partial masculinization including sexual bipotency re-acquisition, follicular degeneration, tubular structure formation, and ectopic SOX9-positive Sertoli-like cell appearance in ovarian grafts in male nude mice. These data with the both mouse models may help elucidate the infertility and masculinization of mammalian fetal ovaries caused by *in utero* exposure to inadequate hormonal conditions, as in the case of human polycystic ovary syndrome and freemartin syndrome in mixed-sex cattle twins.

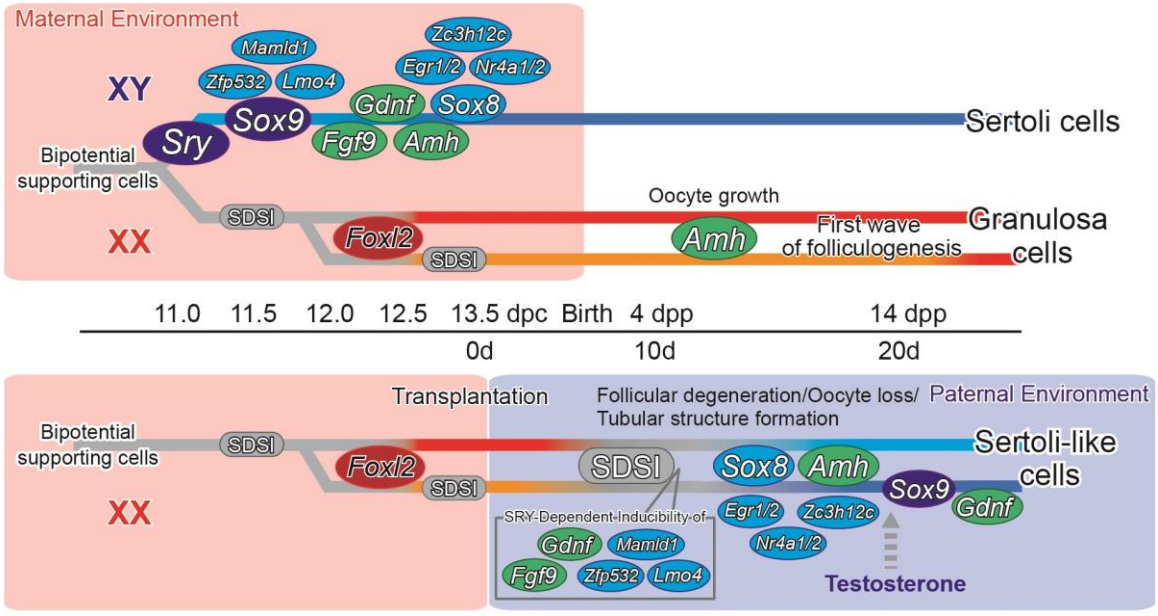

Fig. of General Discussion

Figure of General Discussion

In sex determination of mammalian XY supporting cells (blue bar in "Sertoli cells"), during 11.0–12.0 dpc, SRY promotes SOX9 expression, together with expressions of potential SRY target genes such as *Zfp532*, *Lmo4*, and *Mamld1*, in a male-specific manner, leading to pre-Sertoli cell differentiation (chapter 2). In pre-Sertoli cells, SOX9 induces various testis-specific factors, such as *Fgf9*, *Gdnf*, and *Amh*, leading to Sertoli cell differentiation and testis formation. *Egr1/2*, *Nr4a1/2*, *Zc3h12c*, and other transcription factors show Sertoli cell-specific upregulation during 12.5 dpc (chapter 4). In XX supporting cell differentiation, pre-granulosa cells maintain SDSI until 11.5 dpc. After that stage, a subpopulation of most pre-granulosa cells rapidly loses this ability by 12.0 dpc and comes to express *Foxl2* (upper red bar in "Granulosa cells"). However, another subpopulation of the pre-granulosa cells near the mesonephric tissue maintains SDSI throughout fetal and early postnatal stages, after they come to be FOXL2-positive at fetal stage (lower orange bar in "Granulosa cells"). After birth, both FOXL2-positive subpopulations contribute to the initial round of folliculogenesis together with oocyte-dependent AMH expression (chapter 1), but SDSI in the latter subpopulation is lost by the secondary follicle stage.

Ovarian transplants into male mice show a partial masculinization (bars in "Sertoli-like cells"). Granulosa cells in both subpopulations in grafted ovaries re-acquire sexual bipotency, not only SDSI but also SRY-dependent inducibility of *Fgf9*, *Gdnf*, and potential SRY target genes, by day 10 post-transplantation (chapter 2). Together with expression of *Sox8*, *Amh*, and other transcription factors, grafted ovaries show follicular degeneration and tubular structure formation (chapter 4). In the masculinizing process, host-derived testosterone-dependent *Sox9* and *Gdnf* expression are detected in the medullary region by day 20 post-transplantation (chapter 3), indicating that sexually bipotential pre-granulosa cells in the medullary region in

fetal XX gonads transdifferentiate into Sertoli cell-like cells (lower blue bar in “Sertoli-like cells”).

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