From Sex Determination to Initial Folliculogenesis in Mammalian Ovaries: Morphogenetic Waves along the Anteroposterior and Dorsoventral Axes

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Abstract

Gonadal sex in most mammals is determined based on sex differentiation of the supporting cell lineages. In mouse XY gonads, SRY induces SOX9 upregulation and subsequent FGF9 expression by embryonic day 11.5 (E11.5), leading to the differentiation of Sertoli cells. XX gonads, lacking SRY action, start on the ovarian program through the actions of WNT4 and FOXL2 from around E11.5–12.0. These 2 ovarian factors, together with retinoic acid (RA) action, promote feminization partially through the repression of the masculinizing activities of SOX9, FGF9 and DMRT1. RA initiates meiosis in female germ cell cysts, in which intercellular bridges between interconnected germ cells rapidly undergo cyst breakdown by E17.5. Ovarian morphogenesis is characterized by continuous recruitment of pre-granulosa progenitor cells from the coelomic epithelia during the embryonic stage, which results in the formation of ovigerous cords and tight packing of non-interconnected oocytes (i.e. oocyte nests) at the perinatal stages. At birth, the oocyte nests break

down into single oocytes surrounded by granulosa cells, leading to the assembly of primordial follicles. This review focuses on recent advances in the molecular and cellular events of initial ovarian differentiation, meiotic initiation, germ cell nest breakdown, and primordial follicle formation based on anatomical and morphogenetic aspects.

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Background of Early Gonadogenesis

During early organogenesis of mammalian embryos, the genital ridges arise from a thickening of the coelomic epithelium covering the mesonephric tissue along the posterior trunk around embryonic day 9.5 (E9.5) in mice [Harikae et al., 2013a; Svingen and Koopman, 2013]. In accordance with elongation of the posterior trunk, the bipotential genital ridges as future gonads become evident as a pair of long and narrow structures along an anteroposterior (AP) axis by E10.5 (leftmost image in fig. 1A) [Harikae et al., 2013a; Wainwright et al., 2014]. One of the earliest molecular events in mouse gonadogenesis is an anterior-to-posterior wave-like activation of GATA4 [Hu et al., 2013], which is a zinc finger transcription factor that is essential for the development of gonads

Fig. 1. Schematic representation showing spatiotemporal patterns of major cellular events during mouse ovarian differentiation and development. **A** Gross anatomical changes in the ovary and mesonephros (mes) along the AP and DV axes from E10.0–10.5 to postnatal day 7 (P7). Female germ cells (GC) are shown as red circles, while blue areas indicate gonadal soma. In the left-most plate, arrows represent the migration of PGCs from the hindgut (hg) to the presumptive gonadal region covered by coelomic epithelium (CE). Along the DV axis [i.e. corticomedullary (CM) axis], CE proliferation and its expansion leads to the formation of the cortical region. **B** The horizontal scale represents the developmental stages from

E7.25 to P7. The red, blue, and purple bars represent the cellular events of GCs, somatic cells (i.e. pre-granulosa cells and CE), and morphogenetic soma-GC interactions, respectively. PGC formation at the proximal extraembryonic mesoderm at E7.25 is indicated by a red arrow, while the gray zone shows the critical time window of sex differentiation at E11.0–11.5 (i.e. the sexually bipotential state of gonadal supporting cells) [Hiramatsu et al., 2009]. In the bottom row, the arrows indicate the fate (i.e. first wave of folliculogenesis or dormant cortical follicles) of the pre-granulosa cell progenitors, which may be recruited from CE at each corresponding stage [Mork et al., 2012; Rastetter et al., 2014].

and various other tissues [review by Chlon and Crispino, 2012; Tevosian, 2014]. NR5A1/SF1, a nuclear receptor that is essential for the formation of gonads and adrenals, is also expressed in a similar anterior-to-posterior wave immediately after the onset of GATA4 expression [Schimmer and White, 2010; Hu et al., 2013]. Other potentially critical transcription factors that may help to establish genital ridge formation in mouse embryos include CBX2 [Katoh-Fukui et al., 2012], EMX2 [Kusaka et al., 2010], LHX9 [Birk et al., 2000], and WT1(–KTS) [Hammes et al., 2001; also see reviews by Schlessinger et al., 2010; Eggers et al., 2014].

Because reduced expression of NR5A1/SF1 has been observed in the genital ridges of *Lhx9*-, *Gata4*- and *Cbx2*-null embryos [Birk et al., 2000; Katoh-Fukui et al., 2012; Hu et al., 2013], this receptor appears to be a core transcription factor that acts downstream of these transcriptional networks during early gonadogenesis. Interestingly, a recent study suggested that NR5A1/SF1 may promote the proliferation of gonadal somatic cells partially through its direct regulation of glucose metabolism-related genes [Baba et al., 2014].

Prior to the formation of the gonadal soma starting around E10.0, the primordial germ cells (PGCs) emerge

from the posterior extraembryonic mesoderm at around E7.25 (red arrow in fig. 1B) [Saitou et al., 2002; McLaren, 2003]. After that, PGCs move toward the genital ridges, passing through the dorsal wall of the hindgut using the morphogenetic movement of the hindgut endoderm during E8.0–9.0 [Molyneaux and Wylie, 2004; Hara et al., 2009]. At around E10.5, the PGCs migrate into the genital ridges following SCF-KIT and CXCL12/SDF1-CXCR4 signals [reviews by Molyneaux and Wylie, 2004; Richardson and Lehmann, 2010; Harikae et al., 2013a]. Importantly, however, normal gonadogenesis is not required for PGCs to enter this region in mouse embryos, as evidenced by the fact that PGCs properly migrate into the presumptive gonadal region in the genital ridges of *Lhx9*- and *Gata4*-null mice without any epithelial thickening of the gonadal region [Birk et al., 2000; Hu et al., 2013]. Finally, colonization of the genital ridges by germ cells completes the establishment of the gonadal primordium consisting of germ cells and gonadal somatic cells.

Sex-specific gonadal differentiation is initiated in male gonads from around E11.0, soon after PGC settlement [review by Kanai et al., 2005; Kashimada and Koopman, 2010; Harikae et al., 2013a]. In XY gonads, the sex-determining gene on the Y (*Sry*) is transiently activated for ~8 h in a center-to-pole wave-like manner along the AP axis of the gonadal region [Albrecht and Eicher, 2001; Bullejos and Koopman, 2001, 2005; Sekido et al., 2004; Kanai et al., 2005; Sekido and Lovell-Badge, 2008]. Transient SRY expression directly initiates the upregulation of SRY-related HMG box 9 (*Sox9*) in pre-Sertoli cells, leading to the subsequent expression of FGF9 in a similar center-to-pole wave-like pattern. FGF9 appears to be secreted from the central region toward the poles, which leads to the rapid establishment of high-level expression of SOX9 in the anterior and posterior pole domains in a positive-feedback fashion [Kim et al., 2006; Hiramatsu et al., 2010]. This SOX9-FGF9 positive-feedback system may be involved in the rapid and synchronous testiculogenesis in the long and narrow gonad along the AP axis [review by Harikae et al., 2013a].

In contrast to the simple and straight cascade of the SRY-dependent testis-determining system in early XY gonads, it remains unclear when, where and how XX female gonadal fate is determined and regulated during the fetal and perinatal stages. In this review, we focus on recent advances in the molecular and cellular events of the initial ovarian differentiation and its subsequent development in the fetal and postnatal stages. Moreover, we discuss the dynamics of ovarian differentiation along the AP and dorsoventral (DV) axes and its biological significance as related to anatomical and morphogenetic features.

Initial Molecular and Cellular Events in Ovarian Differentiation

WNT/β-Catenin Signaling in Sex-Dimorphic Proliferation in the Coelomic Epithelial/Subepithelial Region

After E10.5, the coelomic epithelium covering the bi-potential gonad continuously undergoes proliferation, ingression, and/or expansion into the gonadal parenchyma at the ventral side of the mesonephric region [Karl and Capel, 1998; Capel, 2000]. Two key factors secreted by the ovary, wingless-type MMTV integration site family member 4 (WNT4) and R-spondin homolog RSPO1, a ligand of the LGR5 receptor that regulates the WNT/CTNNB1 (β-catenin) signaling pathway [Carmon et al., 2011; de Lau et al., 2011], synergistically regulate the thickening of the coelomic epithelium and its subsequent expansion toward the subepithelial region in both XY and XX gonads [Chassot et al., 2012]. RSPO1/WNT4/CTNNB1 signals have been shown to antagonize the masculinizing factors SOX9 and FGF9 downstream of *Sry* [Kim et al., 2006; Hiramatsu et al., 2009; Jameson et al., 2012; Nicol and Yao, 2015]. In addition to a partial sex reversal in either *Wnt4* or *Rspo1* mutant gonads [Vainio et al., 1999; Jeays-Ward et al., 2003; Jordan et al., 2003; Chassot et al., 2008; Tomizuka et al., 2008], ectopic expression of a stabilized form of CTNNB1 is sufficient to induce male-to-female sex reversal in XY gonads [Maa-touk et al., 2008]. These data are suggestive of multiple important roles of RSPO1/WNT4/CTNNB1 signals from early gonadogenesis to ovarian differentiation.

At the early stages of E10.5~12.5, coelomic epithelial cells proliferate, ingress and expand toward the subepithelial region in both XY and XX gonads, however more evidently in XY gonads [Karl and Capel, 1998; Capel, 2000]. At later stages (~E12.5), the expansion of proliferative epithelial cells continuously occurs only in XX gonads. This is because, after E12.5, somatic cells in the XY gonads are separated from the coelomic epithelium by the testis-specific formation of the tunica albuginea with vasculatures [Karl and Capel, 1998; Mork et al., 2012]. Also considering the high-level activation of canonical WNT/CTNNB1 signaling in the E11.5–12.5 coelomic epithelia in both XY and XX gonads [Chassot et al., 2011, 2012], these data suggest that RSPO1/WNT4/CTNNB1 signals play crucial roles in the expansion of proliferative coelomic epithelial cells in both XY and XX gonads until E12.5. Thereafter, the signals continuously appear to induce expansion of the cortical region only in XX gonads in a sexually dimorphic manner. This in turn indicates that ova-

ry-specific RSPO1/WNT4 signals may regulate the continuous proliferation/expansion of the cortical region in fetal and perinatal ovaries, leading to the continuous recruitment of pre-granulosa cells from the coelomic epithelium, even after birth (fig. 1B, blue bars) [Mork et al., 2012; Rastetter et al., 2014].

In addition to their crucial roles in the proliferation/expansion of the coelomic epithelial/subepithelial regions and their antagonizing action against center-derived masculinizing FGF9 signals, the RSPO1/WNT4 signals promote not only oogonial proliferation and survival but also meiotic initiation of female germ cells in developing ovaries [Liu et al., 2010; Chassot et al., 2011]. In addition, RSPO1/WNT4/CTNNB1 signals promote female-specific expression of various key ovarian genes such as the homeobox gene *Irx3* [Kim et al., 2011], the X-linked nuclear receptor *Nr0b1/Dax1* [Mizusaki et al., 2003] and 2 *Tgfb*-related genes, *Bmp2* [Yao et al., 2004; Kashimada et al., 2011], a potent suppressor of granulosa cell tumors [Pangas et al., 2008], and follistatin (*Fst*) [Yao et al., 2004; Kashimada et al., 2011], an antagonist for activin A, a masculinizing factor [Wu et al., 2013, 2015; Saba et al., 2014b]. The molecular and cellular actions of RSPO1/WNT4 signals, as well as the actions of forkhead box protein L2 transcription factor (FOXL2) and retinoic acid (RA), are summarized in figure 2.

Expression of FOXL2, a Core Ovarian Factor That Directs Granulosa Cell Fate, in a Gradient Manner from the Mesonephric (Dorsal) Side

In developing mouse XX gonads, the initial ovary-specific molecular events occur from around E11.5, ~12 h after the initial onset of Sry expression in the central domain of the XY gonad [Nef et al., 2005; Beverdam and Koopman, 2006; Chen et al., 2012; Munger et al., 2013], albeit with considerable variation among mouse strains, i.e. high-level expression of a large number of ovary-specific genes in the C57BL/6J strain relative to the 129S1/SvImJ strain at the critical time point of sex determination, E11.5 [Munger et al., 2009, 2013]. FOXL2 is one of the early ovarian factors that plays an essential role in the maintenance of ovarian phenotypes of the granulosa cells in developing ovaries [Schmidt et al., 2004; Uda et al., 2004; Ottolenghi et al., 2007; Uhlenhaut et al., 2009]. It is also a crucial female-determining gene in goats [Boulanger et al., 2014]. FOXL2 is expressed in both granulosa cells and steroidogenic theca cells, and it has multiple roles in ovarian development as a direct regulator of CYP19A1, an aromatase that synthesizes estrogens [Panetier et al., 2006; Wang et al., 2007; Sridevi et al., 2012],

and as a repressor of SOX9 and DMRT1 [Uhlenhaut et al., 2009], an evolutionarily conserved DM domain transcriptional factor that is necessary and sufficient for testis differentiation [Matson et al., 2011; Elzaia et al., 2014; Lindeman et al., 2015; Zhao et al., 2015].

In goats, either downregulation or loss of FOXL2 transcription triggers female-to-male sex reversal [Pailhoux et al., 2001; Boulanger et al., 2014]. In mice, *Foxl2*-null XX gonads show no appreciable morphological evidence of sex reversal in fetuses, but do so after birth when transdifferentiation of granulosa cells into Sertoli-like cells is observed [Uda et al., 2004; Ottolenghi et al., 2005; Uhlenhaut et al., 2009]. Moreover, in adult stages, ectopic FOXL2 as well as ectopic ESR1/2 and CTNNB1 drive male-to-female transdifferentiation in *Dmrt1*-null Sertoli cells but not in wild-type Sertoli cells [Minkina et al., 2014]. In mouse XX gonads, FOXL2 expression is first detectable in several gonadal somatic cells located at the anterior mesonephric (anterior-dorsal) side at around E12.0, after which its expression domain rapidly expands toward the coelomic epithelial (ventral) side in addition to the anterior and posterior poles by E12.5 [see fig. S3 in Harikae et al., 2013b]. After E12.5, most of the FOXL2-positive cells reside along the mesonephros, whereas only few are within the coelomic domain [Mork et al., 2012], similar to other genes that display a gradient pattern of ovary-specific expression (e.g. *Slitrk1*, *oncRNA3* and *Egfl6*) in E13.5 ovaries [Chen et al., 2012].

RA Signaling Triggers Meiotic Initiation of Female Germ Cells in an Anterior-to-Posterior Wave-Like Pattern

RA is a crucial regulator of not only the meiotic initiation of female germ cells [Bowles et al., 2006, 2010; Koubova et al., 2006] but also the feminizing effects on supporting cells in developing fetal gonads albeit at postnatal stages [Minkina et al., 2014]. Enhanced RA signaling accelerates transdifferentiation in *Dmrt1*-mutant testes, in which ectopic RA signaling alone can activate genes of female differentiation/maintenance networks via the action of RA receptor alpha (RARA) in postnatal Sertoli cells lacking DMRT1 [Minkina et al., 2014]. Therefore, it is possible that, even during fetal stages, the feminizing signals of RA, as well as WNT/CTNNB1, FOXL2 and estrogen, maintain ovarian differentiation and development, partially by antagonizing the action of DMRT1 [Minkina et al., 2014].

Aldehyde dehydrogenase family member a2/retinaldehyde dehydrogenase 2 (*Aldh1a2/Raldh2*), which encodes an enzyme that synthesizes RA, is highly expressed in the

Fig. 2. Potential interactions and cascades of the major sex-specific transcriptional/signaling factors and cellular events in ovarian differentiation. Germ cells (red frame) and pre-granulosa cells (dark blue frame) in ovarian parenchyma (light blue frame). Black and gray indicate female- and male-specific events/factors/cascades, respectively. CE = Coelomic epithelium.

mesonephric tissues of both XY and XX gonads [Niederreither et al., 2002; Feng et al., 2014], suggestive of a potential major source of RA in developing gonads (fig. 2). In developing gonads, fetal Sertoli cells, but not ovarian somatic cells, highly express CYP26B1, a cytochrome p450 enzyme that degrades RA [Menke and Page, 2002; Bowles et al., 2006; Koubova et al., 2006]. In XX gonads, stimulated by retinoic acid gene 8 (*Stras8*), a meiotic initiation gene potentially induced by RA, is expressed in an anterior-to-posterior pattern starting at E12.5 [Menke et al., 2003; Bowles et al., 2006; Koubova et al., 2006]. *Rec8*, a meiosis-specific gene that encodes a component of the cohesin complex, is also initiated by RA signals in a similar anterior-to-posterior manner independently of *Stras8* function [Koubova et al., 2014]. These data suggest that, at least in fetal germ cells, RA signals act in an anterior-to-posterior manner, and their source appears to be the anterior mesonephric tissues in the ovaries of fetal mice [Bowles et al., 2006]. However, *Aldh1a2/1a3*-null ovaries show proper meiotic induction of female germ cells [Kumar et al., 2011]. Moreover, ALDH1A1 is expressed in germ cells of female mice from E12.5 to E15.5 [Mu et al., 2013], and the expression of the *Aldh1a2* ortholog has been reported in human gonads [Childs et al., 2011] and other vertebrate species [Smith et al., 2008; Piprek et al., 2013]. Therefore, it remains unclear whether the anterior-posterior pattern of meiotic initiation is regulated by diffusible RA signals [Griswold et al., 2012] or by intrinsic

factors such as the RA receptors [Gely-Pernot et al., 2012; Minkina et al., 2014; Ikami et al., 2015] and epigenetic regulators upstream of *Stras8*, such as polycomb repressive complex 1 (PRC1) [Yokobayashi et al., 2013].

Loss of SRY-Dependent SOX9 Inducibility in Initial Pre-Granulosa Cell Differentiation in an Anterior-to-Posterior Wave-Like Fashion

The earliest cellular event in developing ovaries is the loss of SRY-dependent SOX9-inducibility (SDSI) in XX gonadal supporting cells in an anterior-to-posterior wave-like manner from E11.5 to E12.0 [Hiramatsu et al., 2009; Harikae et al., 2013b]. We have previously shown that forced ubiquitous SRY activation throughout the entire region of developing XX gonads at early stages (~E10.0) led to the formation of an XX testis with a normal spatiotemporal SOX9 expression profile, in which the gonadal supporting cells did not show any advance in timing or ectopic activation of *Sox9* initiation [Kanai et al., 2005; Kidokoro et al., 2005]. These data demonstrate a tight regulation of SDSI in gonadal supporting cell precursors and provide a novel approach to estimate sexually bipotential states by monitoring the loss or reacquisition of the potency to initiate SRY-dependent *Sox9* activation in developing ovaries under normal and pathological conditions. Using this novel estimation method with an Sry-inducible transgenic line, spatiotemporal changes in SDSI of ovarian somatic cells were ex-

amed throughout the embryonic and peri- and postnatal stages [Harikae et al., 2013b]. XX pre-granulosa cell precursors maintained the SDSI until E11.5, after which most pre-granulosa cells rapidly lost this ability in an anterior-to-posterior manner by E12.0. At E12.0–12.5, most of the ovarian supporting cells, except for a small population of pre-granulosa cells located at the mesonephric side, completely lost SDSI, indicating that the loss of SDSI is one of the earliest key events in pre-granulosa cell differentiation in developing ovaries starting at around E11.5. Because the anterior-to-posterior loss of SDSI in pre-granulosa cell precursors occurs before the female-specific expression of FOXL2 and independently of WNT4 and RA signals, some intrinsic regulators (e.g. positive/negative regulator of SRY action) may be regulated in XX supporting cells in an anterior-to-posterior fashion during E11.5 to E12.0. One candidate negative regulator of SRY action (anti-SRY factor) is the X-linked NR0B1/DAX1 [Swain et al., 1998], which represses the activation of SOX9, possibly by inhibiting the cooperative actions of SRY and NR5A1/SF1 [Ludbrook et al., 2012]. However, *Nr0b1/Dax1*-null ovaries differentiate contrary to our expectations, with all stages of follicles present except for corpora lutea [Meeks et al., 2003], while genetic males show defective *Sox9* expression [Bouma et al., 2005]. Because *Nr0b1/Dax1* expression is detectable highly and widely in somatic cells of both XY and XX gonads at E11.5–12.5 [Ikeda et al., 2001], the molecular mechanisms of SDSI regulation in gonadal supporting cell precursors require further study.

In summary, in the early phase of ovarian differentiation, the anterior-to-posterior loss of SDSI first occurs in an intrinsic manner from E11.5, similar to the anterior-to-posterior expression patterns of GATA4 and NR5A1/SF1 during the initial gonadogenesis (from ~E10.5). Next, the molecular events of ovarian differentiation in mice appear to be regulated by WNT4 and RA signaling molecules, as well as the FOXL2 transcription factor. FOXL2 expression occurs from the dorsal (mesonephric) side toward the ventral (coelomic epithelial) side from E12.0 in developing mouse ovaries. RA signals appear to be derived mainly from the anterior mesonephric tissues, which results in an anterior-to-posterior pattern of meiotic entry in female germ cells starting at E13.5. WNT4/ β -catenin signals are active in the coelomic epithelial side in both XY and XX gonads at E11.5; in later stages, and only in XX gonads, they expand throughout the whole ovarian parenchyma. The feminizing signals of WNT4, RA and FOXL2 appear to antagonize the intrinsic DMRT1 action in differentiating XX gonads by E13.5 [fig. S10 in Harikae

et al., 2013b], but are competed against and repressed by a center-to-pole masculinizing wave of SRY-dependent SOX9-FGF9 positive-feedback signals in developing XY gonads. The dynamic waves of ovarian differentiation are shown schematically in figure 3.

Continuous Coelomic Epithelial Proliferation Contributes to the Formation of Ovarian Ovigerous Cords along the DV Axis

In developing XY gonads, the testis-specific formation of tunica albuginea and vasculature separate a coelomic epithelium layer from the testicular parenchyma [Capel, 2000], which results in the cessation of coelomic epithelial ingression toward the parenchyma after E12.5. On the other hand, the coelomic epithelium in XX ovaries continuously undergoes proliferation, invagination and expansion [Mork et al., 2012], leading to the formation of ovigerous cords consisting of female germ cell cysts and their surrounding flattened pre-granulosa cells (also called ‘secondary cords’ or ‘ovarian cords’) by perinatal stages. The female germ cells within the ovigerous cord appear to be extended along the DV axis by the perinatal stages. The surrounding supporting cells of these cords are continuously connected with the surface of the coelomic epithelium, but the border between the intra- and extracordal regions shows species-specific variation; for example, well-defined cords form in pigs, sheep and cows, whereas obscure cords form in mice and rats [Byskov, 1986; Sawyer et al., 2002; Mazaud et al., 2005; Hummitzsch et al., 2013, 2015].

Recently, it has become evident that, during fetal to adult stages, coelomic epithelium at the ovarian surface contains epithelial stem cells marked by the expression of LGR5, a specific marker for tissue stem cells in the intestine, skin and hair follicles [Szotek et al., 2008; Flesken-Nikitin et al., 2013; Ng et al., 2014]. LGR5 is a well-known G protein-coupled receptor for R-spondins (RSPO1–4) that are able to enhance WNT/ β -catenin signaling pathways in various morphogenetic processes [review by Schuijers and Clevers, 2012]. Interestingly, after E12.5, LGR5 is highly expressed in XX ovaries and this expression depends on RSPO1/WNT4 signaling [Rastetter et al., 2014]. LGR5-positive cells are restricted to the cortical region of the ovaries throughout the fetal and perinatal stages, and are fated to differentiate into granulosa cells of several growing follicles even in the first folliculogenesis [Rastetter et al., 2014; review by Hummitzsch et al., 2015]. As described above, the proliferation and expansion of

Fig. 3. Schematic representation showing dynamic waves along the AP and DV axes in gonadal somatic and germ cell differentiation during the early phases of sex differentiation. In developing ovaries, the anterior-to-posterior loss of SDSI first occurs in an intrinsic manner from E11.5~12.0 (red arrow). Meiotic entry of female germ cells is also observed with similar anterior-to-posterior patterns, which may be partially caused by the potential diffusion of RA signals from the anterior mesonephros (purple arrow). FOXL2 expression occurs in a dorsal-to-ventral manner at E12.0–12.5 (green arrow), possibly due to the temporal order of the pre-granulosa cell recruitment from coelomic epithelium at E11.0–12.0. In the coelomic epithelium, continuous proliferation and subepithelial expansion leads to the formation of the cortical parts of ovigerous cords, which may be regulated by female-specific WNT4 signals (blue arrows/area). In developing XY gonads, spontaneous activation of such feminizing factors is repressed by a center-to-pole masculinizing wave of a SOX9-FGF9 positive-feedback system downstream of SRY action (gray arrow) at the critical time window (E11.0–11.2) [Hiramatsu et al., 2009]. Testis-specific vasculogenesis from the mesonephros is also shown [Combes et al., 2009; Cool et al., 2011, 2012].

coelomic epithelium is induced by RSPO1/WNT4/CTNNB1 signals in the initial stages of gonadogenesis [Chassot et al., 2012]. This appears to occur continuously in the later stages (i.e. during E12.5–14.5 and until P3 or P4) [Mork et al., 2012]. Therefore, LGR5-positive cells in the cortical region might represent the proliferating cells that lead to the ovary-specific expansion of the coelomic epithelial/subepithelial cortical region throughout the fetal and perinatal stages (fig. 2; also see the blue cells in fig. 4).

Rastetter et al. [2014] demonstrated that LGR5 single-, LGR5/FOXL2 double-, and FOXL2 single-positive pre-

granulosa cells are roughly arranged in spatial order from a ventral (coelomic epithelial)-to-dorsal (mesonephric) side, and they further suggested that the LGR5-positive/FOXL2-negative pre-granulosa cells appear to subsequently differentiate into FOXL2 single-positive pre-granulosa cells through a transient LGR5/FOXL2 double-positive state in developing ovaries. Such a cell-by-cell progressive order of pre-granulosa cell differentiation along the DV axis may reflect the spatial order of pre-granulosa cell recruitment within the ovigerous cords along the DV axis of the developing ovary (fig. 4). In addition, the DV axis-dependent arrangements of such pre-granulosa cell populations are similar to those of 3 pre-Sertoli cell populations in early XY gonads at E11.2–11.5: SRY single-, SRY/SOX9 double-, and SOX9 single-positive pre-Sertoli cells, which are roughly arrayed in spatial order from the ventral (coelomic epithelial)-to-dorsal (mesonephric) side [fig. S2 in Sekido et al., 2004; fig. 6A in Kidokoro et al., 2005; also see review by Harikae et al., 2013a].

Regulation of Meiotic Initiation of Germ Cell Cysts in an Anterior-to-Posterior Manner

After colonization of the PGCs around E10.5, fetal germ cells express DAZL, an RNA-binding protein that is essential for germ cell differentiation from the PGC state to the competent state to respond to somatic cues for gametogenesis in both sexes [Gill et al., 2011]. Simultaneously, they undergo active proliferation (doubling time of about 15–16 h) [Tam and Snow, 1981; Lei and Spradling, 2013] for genome-wide DNA demethylation reprogramming [Seisenberger et al., 2012; Kagiwada et al., 2013], leading to the formation of a germ cell cyst of ~30 germ cell clones per 1 PGC from E10.5 to E14.5 [Lei and Spradling, 2013]. From E12.5, *Stra8* (a gatekeeper gene of meiosis required for pre-meiotic DNA replication) and *Rec8* (a meiosis-specific gene that encodes a component of the cohesin complex) are synchronously activated within the germ cell cyst by RA signals [Bowles et al., 2006; Koubova et al., 2006, 2014], indicative of meiotic entry of female germ cells in an anterior-to-posterior pattern at E13.5–14.5. This is consistent with an opposed posterior-to-anterior pattern of the loss of PGC markers such as *Pou5f1*, *Dppa3/PGC7/Stella* and *Nanos3* in developing ovaries at similar stages [Sato et al., 2002; Menke et al., 2003; Bullejos and Koopman, 2004; Ohinata et al., 2008; Yamaji et al., 2010].

In developing XY gonads, CYP26B1 (an enzyme that degrades RA) and FGF9 (a meiosis-inhibiting factor for

Fig. 4. Formation and reorganization of the ovigerous (OV) cords with germ cell (GC) cysts along the DV axis, leading to the first wave of folliculogenesis in the ovarian medullary region. A subpopulation of FOXL2-positive pre-granulosa cells near the mesonephric tissue continuously retains SDSI throughout the fetal and early postnatal stages (green cells with solid outline in zone i), albeit with a rapid loss of SDSI in most pre-granulosa cell precursors by E12.0. After birth, these SDSI/FOXL2 double-positive pre-granulosa cells contribute to the initial round of folliculogenesis. LGR5-positive pre-granulosa cells are restricted to the cortical region throughout the fetal and perinatal stages (blue cells in zone iv). LGR5 single-, LGR5/FOXL2 double- (dark blue cells in zone iii), FOXL2 single- (green cells in zone ii), and SDSI/FOXL2 dou-

ble-positive cells (green cells with solid line in zone i) are roughly arranged in spatial order from the ventral-to-dorsal side. Female GCs synchronously proliferate and form GC cysts [interconnected GCs; red circles connected by an intercellular bridge (IB)] by E14.5. These GC cysts are tightly packed with pre-granulosa cells within the OV cords extended to the DV axis. By E17.5, the majority of interconnected GCs are broken down into the 1 or 2 connected state (i.e. disruption of IB between interconnected GCs). Sequential processes of primordial follicle (PF) formation (i.e. GC apoptosis, nest breakdown, and the assembly of PFs) occur first in the medullary region and expand to the cortical region in later stages. CE = Coelomic epithelium.

both pluripotency and masculinization of germ cells) repress *Stra8* expression, thereby antagonizing the actions of RA signaling [Bowles et al., 2006, 2010; Koubova et al., 2006] (also see ‘germ cell’ in fig. 2). RSPO1/WNT4 and non-canonical WNT5A signals have been shown to coordinate meiotic initiation of female germ cells in developing ovaries [Vainio et al., 1999; Naillat et al., 2010; Chasot et al., 2011]. In addition, MSX1/2 [Le Bouffant et al., 2011] and DMRT1 [Krentz et al., 2011], as well as DAZL (a germ cell-intrinsic signal response factor) [Koubova et al., 2014] have been shown to be required for proper meiotic entry in female germ cells. NANOS2, an RNA-binding protein, functions as a master regulatory factor for masculinization of XY germ cells, which also represses meiotic initiation through the repression of *Stra8* expression [Suzuki and Saga, 2008; Saba et al., 2014a].

Lei and Spradling [2013] demonstrated that, in mouse XX gonads, germ cells synchronously proliferate within the same clone (cyst), leading to 30 ± 14 cells in 4.8 ± 3.5

cysts at E14.5 (average 5–6 interconnected germ cells via intercellular bridges per cyst). During this period, cyst formation occurs homogenously in the whole ovarian parenchyma. However, female germ cells of each cyst appear to widely expand along the DV axis of the ovigerous cords by E14.5 (fig. 4) [fig. S4 in Lei and Spradling, 2013]. Such synchronized progression in the germ cell cyst extending along the DV axis would explain the anterior-to-posterior expression profiles of *Stra8* and *Rec8* in developing ovaries along the AP axis [Menke et al., 2003; Koubova et al., 2014], even if the meiosis-inducing signals of RA are diffused from anterior mesonephric tissue, one of the major sources of RA synthesis [Bowles et al., 2006]. This is because the intrinsic RA signals in each germ cell at the anterior mesonephric side might be efficiently passed into the other interconnected germ cells positioned at the coelomic epithelial side via the intercellular bridges (see ‘IB’ in fig. 4). Finally, most female germ cells arrest meiosis in the dictyate stage after birth through the

leptotene-to-diplotene stages [Di Carlo et al., 2000; Ghafari et al., 2007]. Such dictyate arrest of oocytes was recently shown to be crucial for the subsequent formation of primordial follicles, as visualized by a novel technique of in situ oocyte chromosome analysis [Wang et al., 2015].

Oocyte Nest Breakdown and the Assembly of Primordial Follicles, Leading to the First Wave of Folliculogenesis from the Medullary Region of the Ovary

Cyst breakdown of the interconnected germ cells, i.e. disruption of intercellular bridges in a germ cell cyst, in developing XX gonads is one of the first cellular events for the assembly of primordial follicles [Pepling and Spradling, 2001]. The interconnected germ cells increase in number to reach a peak at E14.5 around the time of meiotic entry. The germ cell cysts are then gradually broken down into single or double connected germ cells by E17.5, a considerable time before breakdown of the oocyte nest, i.e. an aggregate of non-interconnected oocytes packed within the ovigerous cords, at P0~P2 and the subsequent assembly of primordial follicles at ~P5 (see red and purple bars in fig. 1; fig. 4) [Pepling and Spradling, 1998, 2001; Lei and Spradling, 2013]. During this process, the onset of cyst breakdown has a tendency to first occur in the mesonephric region, followed by those in the cortical region at E14.5 and E17.5 [see fig. S2D in Lei and Spradling, 2013], but there is no significant regional difference in size or cyst number of germ cell clones.

It is generally accepted that the loss of about two thirds of the oocytes accompanies oocyte nest breakdown, as low-quality oocytes are eliminated; this results in the assembly of primordial follicles at the time of birth [Pepling and Spradling, 2001]. Such oocyte loss is induced by developmentally programmed apoptotic pathways [Perez et al., 1999; Alton and Taketo, 2007; Greenfeld et al., 2007], which may be triggered by environmental changes immediately after birth, such as plummeting levels of estrogen [Jefferson et al., 2002; Chen et al., 2007, 2009]. Interestingly, this programmed nest breakdown starts in the medullary region prior to the time of birth, after which the process appears to expand toward the cortical surface region of the ovaries in humans [De Felici et al., 2005; Pepling et al., 2010]. There are similar regional differences in rat ovaries [Mazaud et al., 2005]. In contrast to the onset of germ cell cyst/nest breakdown from the medullary (dorsal)-to-cortical (ventral) side, no appreciable regional differences have been reported in the expression

profiles and the loss-of-function phenotypes of several key oocyte transcription factors such as factor in the germline alpha (FIGLA) [Soyal et al., 2000], newborn ovary homeobox protein (NOBOX) [Rajkovic et al., 2004], spermatogenesis- and oogenesis-specific bHLH transcription factor 1/2 (SOHLH1/2) [Pangas et al., 2006; Choi et al., 2008b], LIM homeobox protein 8 (LHX8) [Choi et al., 2008a], and TATA box binding protein (TBP)-associated factor 4B (TAF4B) [Grive et al., 2014]. Because most oocytes in these loss-of-function mutants ultimately undergo cell death throughout the whole ovarian parenchyma shortly after birth, this homogenous loss-of-oocyte phenotype likely reflects prenatal effects of deficiencies in these transcriptional regulators to produce defective oocytes [review by Jagarlamudi and Rajkovic, 2012]. Further quantitative analyses of the spatiotemporal pattern of the onset of oocyte death and the accurate staging of defective follicular stages in each mutant may be required to understand the roles of each factor in oocyte quality control and/or its interaction with pre-granulosa cells in nest breakdown and subsequent formation of primordial follicles in the perinatal stages.

On the other hand, defective nest breakdown can lead to aberrant follicle assembly resulting in phenomena such as multi-oocyte follicles (MOFs) in postnatal ovaries. MOF phenotypes are also detectable in mutant ovaries and may include several key genes associated with pre-granulosa cell differentiation such as *Wnt4* [Prunskaitė-Hyyryläinen et al., 2014], *Foxl2* [Uda et al., 2004] and *Esr1/2* (estrogen receptor- α/β) [Jefferson et al., 2002]. Such MOF phenotypes may be partially caused by masculinization in affected granulosa cells at the transcriptional level [Garcia-Ortiz et al., 2009]. In addition, the MOF phenotype is observed in various mutants, such as Notch2-Jagged1 signaling factors [Xu and Gridley, 2013; Vanorny et al., 2014], cycline-dependent kinase inhibitor 1 (Cdkn1/p27Kip1) [Perez-Sanz et al., 2013] and water channel aquaporin 8 [Su et al., 2010] [also see review by Pepling, 2012].

Similar to some DV axis-dependent regional differences in cyst/nest breakdown, the primordial follicle assemblies are initially formed preferentially in the medullary region, which engages in the first wave of folliculogenesis at P3–5 [Mork et al., 2012; Zheng et al., 2014]. The granulosa cells in dormant primordial follicles in the cortex region are newly recruited from the coelomic epithelia [Hirshfield, 1992; Hirshfield and DeSanti, 1995; Mork et al., 2012]. During this process, the ovigerous cords become fragmented as the oocytes separate, after which individual oocytes become enclosed in a layer of differenti-

ated granulosa cells from the medullary region at the mesonephric side (dorsal side) toward the surface cortical region (ventral side) [Byskov, 1986; Pepling, 2012] (fig. 4). Lineage tracing studies using a *Foxl2-CreER^{T2}* or *Lgr5-CreER^{T2}* mouse line have shown that most of the pre-granulosa cells in ovigerous cords at E16.5 appear to contribute to primordial follicles in the initial wave of folliculogenesis after birth [Mork et al., 2012; Rastetter et al., 2014]. The first follicles in the ovarian medullary region have several characteristics that differ from the dormant primordial follicle pool in the cortical region: most are fated to undergo atresia at later stages without stimulation from pituitary-derived FSH, they show temporal differences in pre-granulosa cell recruitment from the coelomic epithelia, and their growth takes less time compared to cyclical adult follicles [Mork et al., 2012; Zheng et al., 2014].

During primordial follicle formation, pre-granulosa cells of the medullary follicles are mitotically arrested in fetal stages and then, after birth, enter a mitotically active state [Mork et al., 2012], leading to AMH-positive granulosa cells of the medullary follicles during the initial wave of folliculogenesis [Münsterberg and Lovell-Badge, 1991; Lecureuil et al., 2002; Shinomura et al., 2014]. This suggests that release from mitotic arrest in pre-granulosa cells may be crucial for follicle assembly and the subsequent initial round of folliculogenesis in the medulla. Interestingly, *Wnt4*- and *Rspo1*-null mutant ovaries have been shown to undergo precocious granulosa cell differentiation, together with the loss of germ cells, in an anterior-to-posterior wave manner, which results in ectopic AMH-positive proliferative granulosa cells (albeit without any oocytes) in the anterior part of the fetal ovary [Maatouk et al., 2013]. In addition, certain somatic interactions with female germ cells at pre-meiotic stages are critical to induce precocious pre-granulosa cell differentiation in the anterior part of the *Wnt4*-null ovary [Maatouk et al., 2013], but such regionally distinct phenotypes may be explained, at least in part, by the anterior-to-posterior wave of female germ cell differentiation in such early stages.

Growth differentiation factor 9 (GDF9), which belongs to the TGF β superfamily, is an oocyte-derived signaling factor that is essential for follicular development beyond the primary follicle stage [Dong et al., 1996]. GDF9 upregulates the expression of both the Desert hedgehog (*Dhh*) and Indian hedgehog (*Ihh*) genes in granulosa cells, while DHH and IHH induce the differentiation of GLI1-positive theca progenitor cells during follicle assembly and subsequent folliculogenesis of postnatal ovaries [Liu et al., 2015]. Interestingly, a subpopula-

tion of steroidogenic theca progenitor cells are derived from mesonephric tissues, in which they appear to migrate toward and penetrate the ovarian medulla in perinatal stages. Therefore, it is possible that such theca progenitor cell migration from the mesonephric side partially contributes to the first folliculogenesis that occurs in the medullary region soon after birth.

Most interestingly, SDSI-positive (i.e. sexually bipotential) pre-granulosa cell location is restricted to the ovarian medullary region adjacent to the mesonephros throughout the fetal and postnatal stages [Harikae et al., 2013b] (green cells with solid outline in fig. 4). This sexually bipotential population is mitotically silent throughout the fetal life and then, soon after birth, contributes to the first wave of folliculogenesis in prepubertal stages. This is suggestive of unexpected heterogeneity in the sexual bipotentiality/plasticity of granulosa cells in the first wave of follicles in postnatal ovaries of mice. Moreover, the existence of SDSI-positive granulosa cells involved in the first wave of folliculogenesis may explain the ovotestis-like phenotype in *Esr1/2*- [Couse et al., 1999; Dupont et al., 2003], *Foxl2/Wnt4*- [Schmidt et al., 2004; Uda et al., 2004; Ottolenghi et al., 2007] and *Rspo1*-null ovaries [Chassot et al., 2008; Maatouk et al., 2013], in which transdifferentiation of ovarian follicles into seminiferous-like tubules with SOX9-positive Sertoli-like cells occurs in the central-medullary region during the initial wave of folliculogenesis [Dupont et al., 2003; Ottolenghi et al., 2007]. With regard to these findings, it is likely that in developing ovaries, pre-granulosa cells are heterogeneously arranged along the DV axis of the ovigerous cords in the following order: (i) SDSI/FOXL2 double-positive, (ii) SDSI-negative/FOXL2-positive, (iii) FOXL2/LGR5 double-positive, and (iv) FOXL2-negative/LGR5-positive populations of the pre-granulosa cells from the mesonephric (dorsal) to coelomic epithelial (ventral) side (see right edge in fig. 4). Such spatial ordering allows for the recruitment of the developing follicles in a DV axis-dependent manner after birth, leading to the first folliculogenesis from ~P2–3.

Conclusion and Future Perspectives

In this review, we focused mainly on recent findings regarding molecular and cellular events of pre-granulosa cells from initial ovarian differentiation along the AP axis to the first folliculogenesis along the DV axis, and discussed the dynamics and biological significance of ovarian differentiation/development in terms of anatomy and

morphogenesis. Briefly, initial gonadogenesis, marked by e.g. GATA4/NR5A1 expression, occurs in an anterior-to-posterior wave-like manner, leading to dynamic waves along the AP axis in gonadal somatic and germ cell differentiation during the early phases of ovarian differentiation. Such AP-axis waves appear to be maintained at least in part by the ovigerous cords of the germ cell cysts (i.e. interconnected germ cells extended along the DV axis) by E14.5. After meiotic progression and oocyte cyst breakdown, such anterior-to-posterior waves are no longer observable and instead DV axis-dependent morphogenetic waves, which are produced by the proliferation/expansion of the coelomic epithelial/subepithelial region at the ventral (cortical) side in addition to a cellular/signaling contribution from the dorsal (medullary) side, become apparent.

At this time, the molecular mechanisms of the initial AP-axis-dependent pre-granulosa cell differentiation (i.e. loss of SDSI) and the assembly of primordial follicles from the medullary side in the perinatal stages remain unclear, as does the significance of the initial wave of folliculogenesis from the medullary side. To resolve these questions, one approach is to identify and characterize anti-SRY factors that may cause the loss of SDSI just after the critical time window of sex determination in mice. This would clarify the function and biological significance of SDSI-positive granulosa cells located in the med-

ullary region, which contributes to the initial round of folliculogenesis in postnatal ovaries. We have developed an AMH-treck mouse line, which allows us to deplete AMH-positive follicles in neonatal ovaries by treatment with diphtheria toxin [Shinomura et al., 2014], leading to small aberrant ovaries with a drastic reduction in the 3 β -HSD-positive interstitial region during the peripubertal stages. Further detailed analyses of the phenotypes of the ovaries missing the initial round of folliculogenesis should increase our understanding of the significance of the first wave of folliculogenesis from a reproductive biological aspect. Moreover, advanced approaches such as quantitative whole-mount imaging of single cells within intact ovaries [Faire et al., 2015] and mathematical simulation of spatiotemporal changes [Da Silva-Buttkus et al., 2009] may increase our understanding of the spatiotemporal dynamics in ovigerous cords and subsequent remodeling of follicle assembly in mammals.

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