Ovarian Organogenesis along the Cortical–Medullary Axis in Mammals

Subjects: Developmental Biology

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In most mammals, the sex of the gonads is based on the fate of the supporting cell lineages, which arises from the proliferation of coelomic epithelium (CE) that surfaces on the bipotential genital ridge in both XY and XX embryos. Genetic studies and single-cell transcriptome analyses in mice have revealed the cellular and molecular events in the two-wave proliferation of the CE that produce the supporting cells. This proliferation contributes to the formation of the primary sex cords in the medullary region of both the testis and the ovary at the early phase of gonadal sex differentiation, as well as to that of the secondary sex cords in the cortical region of the ovary at the perinatal stage. To support gametogenesis, the testis forms seminiferous tubules in the medullary region, whereas the ovary forms follicles mainly in the cortical region. The medullary region in the ovary exhibits morphological and functional diversity among mammalian species that ranges from ovary-like to testis-like characteristics.

sex differentiation testis ovary folliculogenesis cortex medulla

1. Early Gonadal Supporting Cell Development in Mice

The sex of most mammals is determined by a pair of sex chromosomes at fertilization—females are XX and males are XY—whereas the genital organs of both females and males are developed from the common genital primordium. The testis in males and the ovary in females both arise from the embryonic gonad to support gametogenesis and sex hormone production. In the testis, sperm are produced in the convoluted seminiferous tubules, of which the epithelia consist of spermatogenic cells and somatic supporting cells called Sertoli cells. In the interstitium between the seminiferous tubules harbors, Leydig cells, which are the steroidogenic cells responsible for the secretion of testosterone, are present [1]. In the ovary, an egg is developed in the follicle that consists of an oocyte and a layer of somatic supporting cells called granulosa cells. Surrounding the growing follicle, a layer of theca cells, which are the steroidogenic cell lineage that produces androgen, is formed [2]. Spermatogenic cells, Sertoli cells, and Leydig cells in the testis are the counterparts of oocytes, granulosa cells, and theca cells in the ovary, respectively [3]. The fates of these cell lineages stem from the bipotential precursors in the gonadal primordium according to the sexually dimorphic genetic programs corresponding with the chromosomal sex of an individual.

(1) Origin of Gonadal Supporting Cells from the Coelomic Epithelium

In mice, the gonadal primordium (also known as the genital ridge) arises from the thickening of the coelomic epithelium (CE) on the ventral surface of the mesonephros around embryonic day 9.5 (E9.5) [4][5][6][7][8]. These

bipotential gonadal primordia become evident as a pair of long and narrow structures along the anteroposterior (AP) axis by E10.5 (**Figure 1**A) [6][9]. The gonadal primordium develops as a result of proliferation, epithelial–mesenchymal transition, and the migration of the CE cells into the dorsal inner layers of CE via its basal lamina. Cell lineage tracing experiments indicate that CE cells are a source of somatic cell precursors, and the cells derived from the CE migrate into the medullary region, forming the primary sex cords in male and female gonads (**Figure 1**A) [10]. Asymmetric cell division and the ingression of CE cells into the gonad require the proper cell polarization of CE cells. In this process, the localization of NUMB (the monomeric PTB-containing adaptor protein, *Numb*), an antagonist of Notch signaling, plays an important role in establishing cell polarity in CE cells (**Figure 1**A) [11].

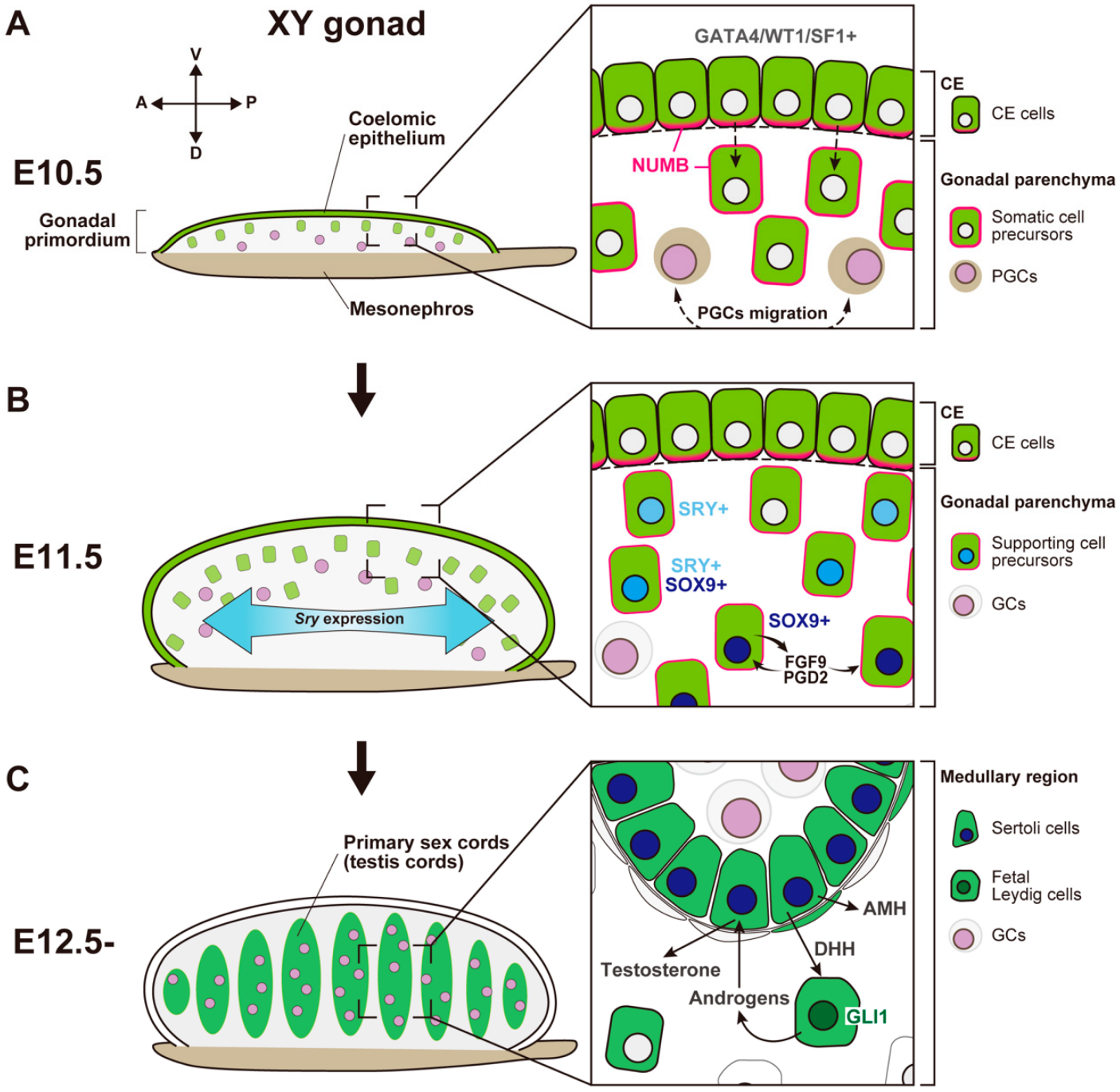

Figure 1. Schematic representation showing morphological change and somatic cell differentiation during the sex determination period, from the bipotential gonadal primordium at E10.5 to the differentiated testis at E12.5. (**A**) At E10.5, the gonadal primordium appears as a long and narrow structure composed of the coelomic epithelium (CE) and the migrated CE cells (somatic cell precursors). CE cells express GATA4/WT1/SF1, and their ingression and asymmetric cell division are primarily controlled by Notch signaling with the NUMB distribution (magenta) and Notch signaling. PGCs migration occurs around E10.5. (**B**) By E11.5, the gonadal primordium expands along the dorsoventral axis via the ingression of CE cells. Testis-specific *Sry* expression occurs in a center-to-pole manner along the anteroposterior axis. Beneath the CE, supporting cell precursors, SRY single- (cyan nuclei), SRY/SOX9 double- (blue nuclei), and SOX9 single- (deep-blue nuclei) positive cells, are distributed from the CE (dorsal)-to-mesonephric (ventral) side. SOX9-positive Sertoli cells secrete FGF9 and PGD2, and these factors upregulate and maintain *Sox9* expression in the own and neighboring supporting cell precursors. (**C**) At E12.5, testis cords are formed by Sertoli cells and germ cells (GCs). SOX9-positive Sertoli cells secrete paracrine factors, such as AMH and DHH. DHH signaling activates its downstream factor, GLI1, in fetal Leydig cell progenitors. Fetal Leydig cells with activated GLI1 (deep-green nuclei) produce androgens, which are converted to testosterone by Sertoli cells and then induce proper differentiation of the internal and external genital tract.

(2) SRY-Mediated Primary Sex Determination

In the XY gonad, the gonadal primordium undergoes sex-specific differentiation soon after the arrival of PGCs [6][12][13]. The sex-determining region of the Y chromosome (*Sry*), which encodes a high-mobility group (HMG)-domain transcription factor, initiates the differentiation of supporting cell precursors into Sertoli cells within the primary sex cords [14][15]. Since its discovery, *Sry* has been regarded as a single-exon gene. However, a recent study revealed that mouse *Sry* has a cryptic second exon and the SRY protein has two distinct isoforms, the canonical SRY-S isoform and a novel SRY-T isoform [16]. SRY-S has a degron at the C-terminus, which results in its rapid degradation in vivo. In contrast, SRY-T is stably expressed in the gonads because of the absence of the degron. Furthermore, the ectopic expression of SRY-T can lead to female-to-male sex reversal in XX mice [16]. Thus, SRY-T isoform is regarded as the determinant of male fate.

Sry is transiently expressed in supporting cell precursors at E10.5–12.5. *Sry* expression is first detectable at the center of the gonad at E10.5 and extends toward cells at the anterior and posterior ends by E11.5 (**Figure 1**B) [17][18]. This *Sry* expression is rapidly downregulated, such that it disappears around E12.5. The loss of *Sry* causes complete XY gonadal sex reversal of the fetal and adult gonads [19].

The principal target of SRY is SRY-related HMG box 9 (*Sox9*), a critical testis-determining factor [20]. In XY gonads, SRY single-positive cells, SRY/SOX9 double-positive cells, and SOX9 single-positive cells are distributed in spatial order from the cortical (coelomic epithelial) side to the medullary (mesonephric) side (**Figure 1**B) [21][22]. The loss of *Sox9* also induces complete gonadal sex reversal. Therefore, SOX9 expression is necessary for the specification of the Sertoli cell lineage [23]. In Sertoli cell precursors, SOX9 expression induces male-specific expression of fibroblast growth factor 9 (*Fgf9*), and FGF9 promotes the high expression of SOX9. These signals form a positive-regulatory feedback loop and maintain each other's expression (**Figure 1**B) [24]. FGF9 expression exhibits a

center-to-pole wave-like pattern, which acts as a diffusible inducer and establishes high expression of SOX9 to induce Sertoli cell fate [25]. SOX9 expression also induces the expression of prostaglandin D2 synthase (brain) (*Ptgds)* [26]. Then, synthesized prostaglandin D2 (PGD2) from SOX9-positive Sertoli cells acts as an autocrine/paracrine factor and amplifies SOX9 signaling in a manner independent of FGF9 (**Figure 1**B) [26][27].

Gene expression profiling at a single-cell resolution can be used to distinguish various cell populations in an unbiased manner. The cell lineage can be predicted based on time-series sampling data via the reconstruction of developmental trajectories; these data provide insights into somatic cell differentiation during testis and ovary development. In mice, in accordance with in vivo and in vitro lineage tracing experiments, developmental trajectory analysis based on single-cell RNA sequencing (scRNA-seq) revealed that proliferating CE cells are the primary source of gonadal somatic cells for both XY and XX gonads [28][29]. A study based on scRNA-seq showed that a new uncharacterized somatic cell population, supporting-like cells (SLCs), contributes to the formation of rete testis and rete ovarii [30]. Rete testis is an anastomosing canal connecting the seminiferous tubules and the epididymis [31]; rete ovarii is a group of anastomosing tubules located in the hilum of the ovary [32]. SLC lineage is the first distinct somatic cell lineage specified in the bipotential gonads, as early as E10.5, before the initiation of gonadal sex determination. SLC progenitors initially express both *Wnt4* and *Sox9*, and they become sexually dimorphic around E12.5. Later, gene expression in SLCs becomes more Sertoli-like or granulosa-like state and mainly contributes to the formation of rete testis or rete ovarii, respectively [30].

Testicular cords, which develop into the seminiferous tubules in the mature testis, are formed via the aggregation of Sertoli cells and germ cells in the medullary region of XY gonads by E12.5 (**Figure 1**C). After E11.5, the proliferation of CE cells is induced in response to platelet-derived growth factor (PDGF) in XY gonads. The cells proliferating during E11.5–E13.5 only differentiate into interstitial cells, resulting in a rapid increase in testis size [33].

Differentiated Sertoli cells produce various autocrine/paracrine signals, which induce the differentiation of other testicular cell populations. For this reason, the proper establishment of Sertoli cells depending on the expression of *Sry* and its downstream gene cascade is thought to be a crucial event in sex differentiation in the testis. Anti-Müllerian hormone (AMH), a paracrine factor secreted by SOX9-positive supporting cells, induces the regression of the Müllerian duct, a primordium of the female reproductive tract (**Figure 1**C) [34][35]. The loss of functional AMH or its receptor AMHR2 results in males with a female reproductive tract derived from the Müllerian duct in mice and humans with a fully virilized phenotype; this condition is known as Persistent Müllerian duct syndrome (PMDS) [35][36][37][38][39]. AMH secreted by Sertoli cells decreases the size of the Leydig cell population [35]. Desert hedgehog (DHH) is a paracrine factor secreted by differentiated Sertoli cells; the expression of DHH is induced by the expression of SRY and SOX9 [40][41]. DHH binds to its receptor PTCH1 in the interstitial progenitor cell population and upregulates gene expression by activating its downstream transcription factor, GLI1 (**Figure 1**C).

2. Molecular and Cellular Events in Ovarian Somatic Cells

(1) Female Fate Determination in Somatic Supporting Cells in the Early Phase of Ovarian Development

At the early phase of ovarian development, granulosa cells are differentiated from common somatic precursor cells with Sertoli cells in the gonadal primordium [17]. Around E10, CE cells covering the gonadal primordium proliferate and ingress into the gonadal parenchyma at the ventral side of the mesonephric region, leading to the formation of a primary sex cord (**Figure 2**A) [3][10]. Wingless-type MMTV integration site family member 4 (WNT4) regulates the thickening of the CE and its expansion toward the subepithelial region in both XX and XY gonads [42]. In conjunction with R-spondin homolog 1 (RSPO1), a ligand of the leucine-rich repeat-containing G-protein-coupled receptors LGR4 and LGR5 regulate the WNT4/β-catenin signaling pathway [43]. At E11.25–11.5, *Wnt4* and *Rspo1* are expressed, and β-catenin signaling is activated in the coelomic region of both XX and XY gonads. Thus, RSPO1/WNT4/β-catenin signaling does not exhibit sexual dimorphism at the beginning of the gonadal sex differentiation process [42]. Around E11.5, 12 h after the initiation of *Sry* expression in the central region of the XY gonads [18][22], the XX gonadal primordium promotes ovarian differentiation by inducing pro-ovarian genes and repressing pro-testis genes [44][45][46]. From E11.5, the activation of RSPO1/WNT4/β-catenin signaling in XX gonads promotes the expression of pro-ovarian genes such as the X-linked nuclear receptor *Nr0b1*/*Dax1* [47], follistatin (*Fst* [48]), Tgfb-related genes, bone morphogenetic protein 2 (*Bmp2* [48]) and the homeobox gene *Irx3* [49]. RSPO1/WNT4/β-catenin signaling in the ovary also promotes the proliferation and survival of oogonia, as well as the meiotic initiation of female germ cells [50][51]. Furthermore, RSPO1/WNT4/β-catenin signaling mediates ovarian development by antagonizing the masculinizing factors SOX9 and FGF9, which are downstream of SRY [24][25][52]. XX gonads in *Wnt4* or *Rspo1* mutant mice show partial sex reversal, including the upregulation of *Sox9* and the appearance of testicular vasculature [53][54][55][56][57].

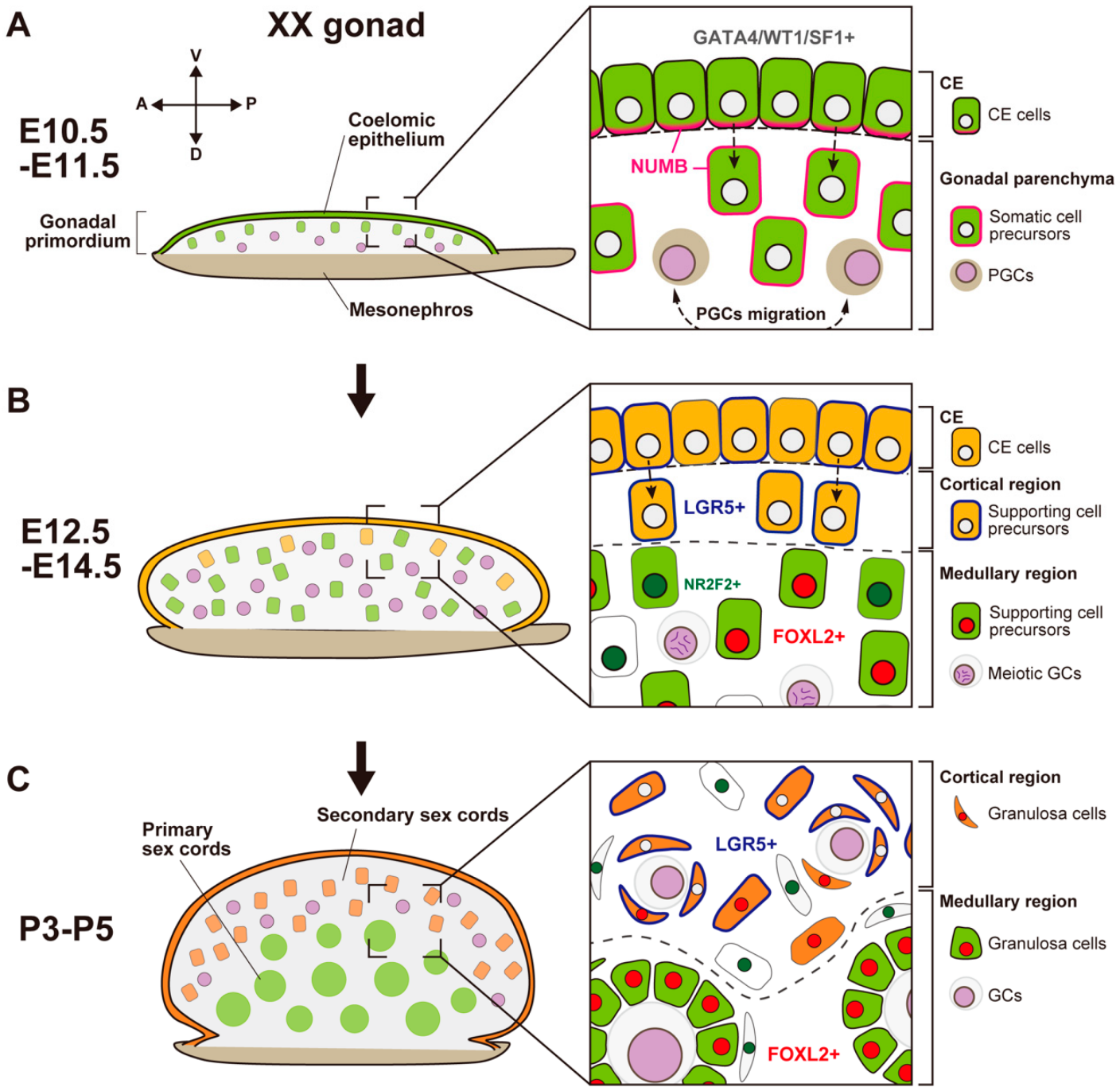

Figure 2. Schematic representation showing morphological changes and somatic cell differentiation along the cortical-medullary axis during fetal and postnatal stages, from the bipotential gonadal primordium at E10.5 to the differentiated ovary around P5. (**A**) At E10.5, the CE cells proliferate and ingress into the gonadal parenchyma, controlled by the NUMB distribution (magenta) and Notch signaling, to form primary sex cords without sexual dimorphism. (**B**) At E12.5, supporting cell precursors in the medullary region (green cells) express an early ovarian factor, FOXL2 (red). In contrast to testis differentiation, the proliferation and ingression of the CE in XX gonads continues after E12.5, leading to the formation of secondary sex cords in the cortical region (orange cells). Supporting cell precursors in secondary sex cords, including CE cells, express the transmembrane receptor LGR5 (blue). LGR5 and FOXL2 expression is mutually exclusive, and it exhibits a gradient from the cortex (CE side) to

the medulla (mesonephric side). Other somatic cells (i.e., interstitial precursor cells) also exclusively express NR2F2 (deep green). Germ cells (GCs) initiate meiosis at E12.5 (deep purple, condensed chromosomes). (**C**) At postnatal stages, FOXL2-positive granulosa cells in primary sex cords in the medullary region contribute to the formation of the first wave of follicles. In the cortical region, granulosa cells that originate from LGR5-positive cells (orange cells) form the secondary sex cords and primordial follicle pools. After sexual maturity, these follicles develop according to the estrous cycle.

In mouse XX gonads, the ovarian-specific transcriptional program begins around E11.5 [45][58][59][60]. Forkhead box L2 (FOXL2) is an early ovarian factor that is essential for the fate and phenotype of granulosa cells [61][62][63][64]. FOXL2 expression is induced later than the activation of the WNT4/β-catenin pathway [45]; the loss of *Ctnnb1* (β-catenin) decreases FOXL2 expression, whereas the stabilization of β-catenin in XY gonads induces FOXL2 expression [65]. These results suggest that RSPO1/WNT4/β-catenin signaling is essential for FOXL2 upregulation in granulosa cells.

There are functional differences between mice and other mammalian species in relation to the role of *Foxl2* and related genes in ovarian development. In goats, FOXL2 is a key female sex-determining gene [66][67]. *FOXL2* is a gene responsible for polled intersex syndrome (PIS), which leads to XX female-to-male sex reversal associated with the absence of horn growth [67]. In the fetal XX gonads of *FOXL2* knock-out goats, *SOX9* and *DMRT1* are upregulated in Sertoli-like cells, forming testicular cords [66]. In mice, in contrast, the loss of *Foxl2* in XX gonads does not induce an appreciable sex-reversal phenotype at the fetal stage and birth, although it results in the transdifferentiation of granulosa cells into Sertoli-like cells in the postnatal stage [62][64][68].

(2) Secondary Population of Granulosa Cells in the Cortical Region of the Ovary

In contrast to mouse XY gonads, the CE in XX gonads continuously exhibits proliferation, ingression, and expansion [69]. This contributes to the formation of ovigerous cords that consist of female germ cells and the surrounding pregranulosa cells by the perinatal stage. These cords are regarded as secondary sex cords or ovarian cords (**Figure 2**B).

In the fetal to adult stages, LGR4 and LGR5, receptors for RSPO1, are expressed in the cortical region of XX gonads, including the proliferative region of the ovarian surface epithelium (**Figure 2**B) [70][71]; the expression of LGR5 after E12.5 is dependent on RSPO1/WNT4/β-catenin signaling [71]. LGR4 and LGR5 are markers for tissue stem cells, such as stem cells in the mammary gland, intestine, and hair follicle [72][73][74][75]. LGR4 and LGR5 are also markers of stem/progenitor cells in the ovarian surface epithelium, which generate new granulosa cells that contribute to cortical follicles until birth (**Figure 2**B) [71][76]. In the postnatal ovary after the cessation of granulosa cell recruitment, *Lgr5* is restricted to stem cells in the ovarian surface epithelium; moreover, *Lgr5* promotes the regenerative repair of ovulatory wounds in the adult ovary [70]. In both XX and XY gonads, RSPO1/WNT4/β-catenin signaling is involved in the proliferation of the CE in the early stage of gonadogenesis [42]. In contrast, the expression levels of LGR4 and LGR5 are higher in XX gonads compared with XY gonads after E12.5; this timing coincides with the ovarian-specific proliferation of the CE throughout the fetal and perinatal stages (**Figure 2**B,C).

(3) Cortical–Medullary Regionality of Folliculogenesis Waves

In mice, PGCs move to the gonadal primordium around E10.5, where they undergo active proliferation, which leads to the formation of germ cell cysts by E14.5 [77][78]. One germ cell produces approximately 30 cell clones via proliferation, which results in the formation of an average of 4.8 cysts that comprise five or six germ cells connected by an intercellular bridge [78]. This cyst formation occurs homogenously throughout the ovarian parenchyma, and the cysts are organized into ovigerous cords along the cortical–medullary axis by E14.5 [78]. Beginning at E12.5, meiosis in female germ cells in mouse XX gonads is initiated by retinoic acid signaling from anterior mesonephric tissue (**Figure 2**B) [79][80], which is activated by stimulated by retinoic acid 8 (*Stra8*) and REC8 meiotic recombination protein (*Rec8*) in an anterior-to-posterior manner [81][82].

In mouse XX gonads, the cyst breakdown of the interconnected germ cells begins in the medullary region, followed by the cortical region from E14.5 to E17.5 [78][83]; the cysts then become single or double-connected germ cells. Furthermore, some oocytes are eliminated via apoptosis, mainly in the medullary region, which results in the enrichment of germ cells in the cortical region [84][85][86]. In the perinatal stage, a surviving germ cell is surrounded by a layer of squamous somatic supporting cells, which results in the formation of a primordial follicle. Primordial follicles undergo folliculogenesis, ending with either ovulation or follicular death (i.e., atresia). The stages of folliculogenesis are (1) primary follicles with a single layer of granulosa cells surrounding the oogonia, (2) secondary follicles consisting of a stratified epithelium of granulosa cells surrounding the oogonia, and (3) tertiary follicles with a well-developed central cavity called an antrum. In mice, the first wave of folliculogenesis occurs in the medullary region soon after birth, and the pregranulosa cells in the medullary region contribute to the first follicles (**Figure 2**C) [69][76]. Subsequently, a wave of folliculogenesis occurs in the cortical region, which harbors the granulosa cells that are newly recruited from stem/progenitor cells in the LGR5-positive ovarian surface epithelium (**Figure 2**C) [71][76].

3. Diversity of Ovarian Organogenesis along the Cortical–Medullary Axis

In the testis developmental pathway, the male-specific gene cascade (SRY, SOX9, and AMH) is highly conserved among mammals. In contrast, ovarian organogenesis—such as the formation of secondary sex cords, the initiation of meiosis in germ cells, and the timing of folliculogenesis—exhibits considerable diversity among mammalian species. To understand this diversity, it may be helpful to focus on the regionalization of the medullary region (the primary sex cords) and the cortical region (the secondary sex cords), which correspond to the region of future folliculogenesis in the ovary (**Figure 3**A). Indeed, the morphological characteristics of the ovarian medullary region exhibit considerable diversity among mammal species.

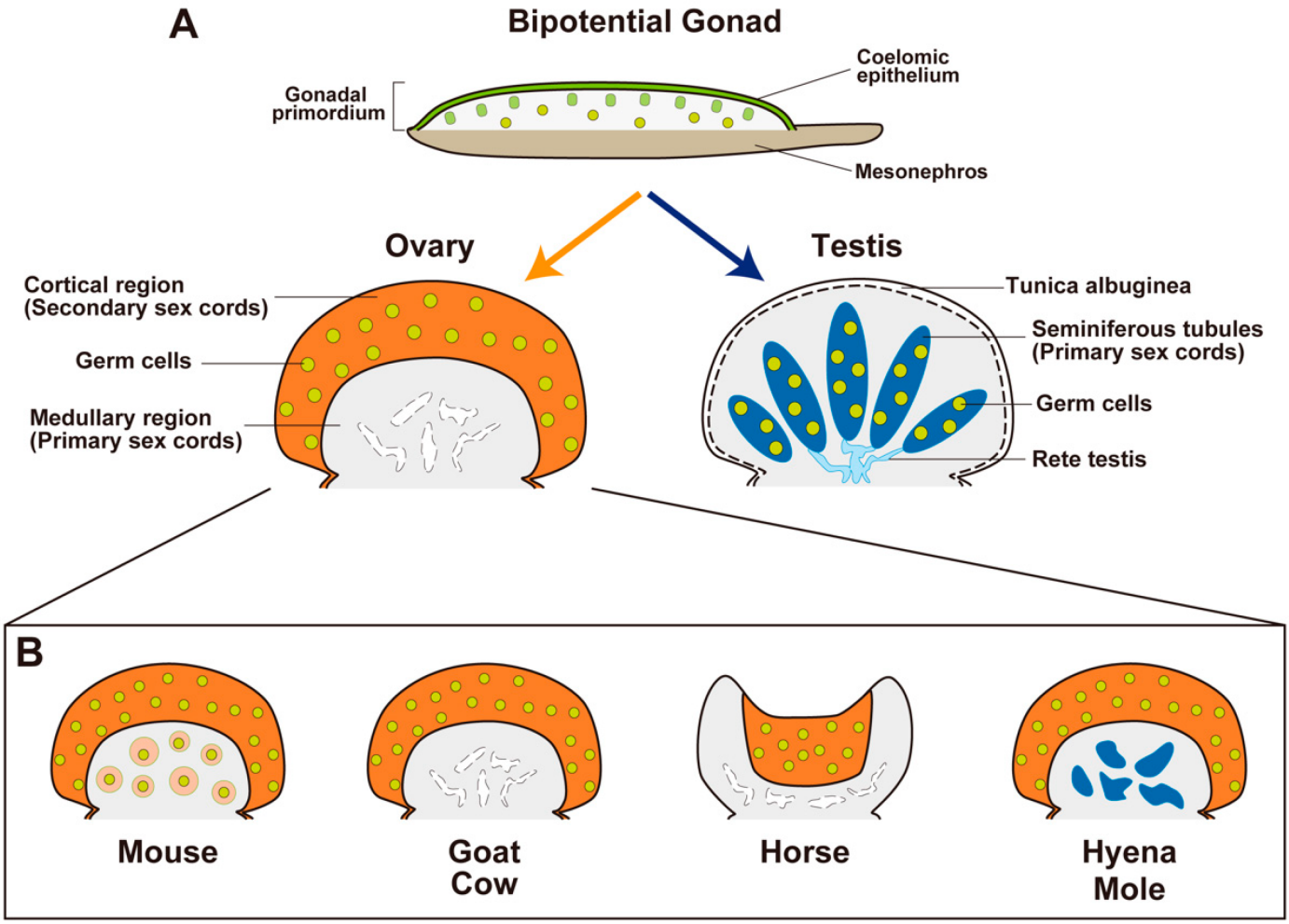

Figure 3. Sexual differentiation of bipotential gonads and the histological variations of ovaries in the indicated species. (**A**) Bipotential gonads differentiate into testes or ovaries. In mammalian testes, primary sex cords form tubular structures (seminiferous tubules (deep blue) with germ cells (yellow dots)) and the rete testis (cyan), which provide a route for continuous sperm transportation from the seminiferous tubules to the efferent ducts. Interstitial components are shown in gray. Mammalian ovaries commonly develop secondary sex cords with germ cells in the cortical region as a site of future folliculogenesis (orange; yellow dots, germ cells). However, the morphological characteristics of primary sex cords in the medullary region of the ovary show considerable diversity among mammals. (**B**) In the ovaries of goats and cows, secondary sex cords in the cortical region form during the fetal period. Germ cells in the medullary region disappear and the primary sex region regresses. In the ovaries of mice, secondary sex cords in the cortical region are not distinct in the fetal period; germ cells in the medullary region are maintained and develop immediately after birth as the first wave of follicles (pale-orange circles with yellow dots). In the ovaries of spotted hyenas and most mole species, the medullary region develops as a male-like tissue with Leydig-cell-like steroidogenic cells (deep blue). In the ovaries of horses, the cortical region (in which folliculogenesis occurs) is surrounded by the well-developed medulla and forms a unique structure known as the ovulation fossa.

In the ovaries of goats and cows, species with a longer gestating period (*Capra hircus*, 150 days; *Bos taurus*, 280 days), distinct secondary sex cords in the cortical region are formed during the fetal period; folliculogenesis occurs before birth. In contrast, the few germ cells in the medullary region immediately disappear during the fetal period, and the primary sex cords regress (**Figure 3**B) [87]. Horses (*Equus caballus*) have large developed medullary region in the ovary. The equine ovary has a unique structure that includes a concave cortical region known as the ovulation fossa [2][87]. The cortical region is restricted to the central area enclosed within a dense, richly vascularized connective tissue casing, which corresponds to medulla [88]; folliculogenesis is limited to the central cortical region (**Figure 3**B). In some mammalian species, ovarian development exhibits a unique pattern, with an ovotestis-like structure containing ovarian tissue in the cortical region and a testis-like structure in the medullary region. In the ovary of the spotted hyena (*Crocuta crocuta*; gestation period, 110 days), the medullary region is separated from the cortical region by a connective tissue boundary during the mid-gestation period. In the medullary region, a cluster of cells expresses 3βHSD; these cells are regarded as Leydig cell-like steroidogenic cells (**Figure 3**B) [89]. In contrast, AMH expression is not present in the cortical or medullary region, suggesting that the supporting cells are not masculinized [89]. In the ovaries of most species of moles, secondary sex cords are formed earlier than the testicular cords in male moles [90]. Additionally, a large medullary region develops that encompasses Leydig-like cells and a testicular cord-like structure [90]; this structure does not exhibit the expression of SOX9 or AMH (**Figure 3**B) [91].

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