Chapter 1
Early Development of the Gonads: Origin and Differentiation of the Somatic Cells of the Genital Ridges

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Abstract The earliest manifestation of gonadogenesis in vertebrates is the formation of the genital ridges. The genital ridges form through the transformation of monolayer coelomic epithelium into a cluster of somatic cells. This process depends on increased proliferation of coelomic epithelium and disintegration of its basement membrane, which is foreshadowed by the expression of series of regulatory genes. The earliest expressed gene is *Gata4*, followed by *Sf1*, *Lhx9*, *Emx2*, and *Cbx2*. The early genital ridge is a mass of somatic SF1-positive cells (gonadal precursor cells) that derive from proliferating coelomic epithelium. Primordial germ cells (PGCs) immigrate to the coelomic epithelium even in the absence of genital ridges, e.g., in mouse null mutants for *Gata4*. And conversely, the PGCs are not required for the formation of the genital ridges. After reaching genital ridges, the PGCs become enclosed by somatic cells derived from coelomic epithelium. Subsequently, the expression of sex-determining genes begins and the bipotential gonads differentiate into either testes or ovaries. Gonadal precursor cells, derived from coelomic epithelium, give rise to the somatic supporting cells such as Sertoli cells, follicular cells, and probably also peritubular myoid and steroidogenic cells.

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1.1 The Earliest Events of Gonadogenesis

The gonads initially form as sexually bipotential structures termed the genital ridges (gonadal ridges). Such precursors of testes and ovaries arise at the ventromedial surface of the embryonic kidneys (mesonephroi) as twofolds running along both sides of the dorsal mesentery (Brambell 1927; Gropp and Ohno 1966; Pelliniemi 1975; Wartenberg et al. 1991). The formation of the primordial gonads starts with the increasing proliferation of the coelomic epithelial cells within a strictly defined site. This leads to the transformation of a monolayer epithelium into a dense and pseudostratified layer underlined by the basement membrane that subsequently disintegrates (Fig. 1.1a, b) (Hu et al. 2013; Karl and Capel 1998; Kusaka et al. 2010; Paranko 1987). Due to disintegration of the basement membrane, the epithelial cells are able to migrate inward and form multilayered structure, whose cells undergo epithelial–mesenchymal transition (EMT). The quintessence of the genital ridges formation is the differentiation of the monolayer coelomic epithelium into multilayered thickening to which the primordial germ cells immigrate. The molecular mechanisms driving coelomic epithelium transformation into the genital ridge remain unclear.

In mice, the first morphological sign of the genital ridge formation—appearance of the coelomic epithelium thickening and disintegration of the basement membrane underneath—is noticeable at 10.3–10.4 *dpc* (days *post coitum*), i.e., stage 5–6 ts (tail somite stages) (Hu et al. 2013). Kusaka et al. (2010) showed that as early as at 10.25 *dpc*, the coelomic epithelial cells are already able to migrate and begin formation of thickened gonadal anlage. In the literature there is a discrepancy in the timing of genital ridge formation onset, which may reflect diversity in the rate of mouse development. Some studies indicate that the genital ridges begin development at 9.0, 9.5, 10.0, or 10.5 *dpc* (Chen et al. 2012; Hacker et al. 1995; Karl and Capel 1995; Nef and Parada 2000; Tanaka and Nishinakamura 2014). Thus, the onset of gonadal development occurs soon after the intermediate mesoderm starts to differentiate into the pronephros and mesonephros, which takes place at 9.5 *dpc*. Just after 10 *dpc*, the genital ridges start to develop at the surface of the anterior part of the mesonephroi. It has been postulated that the gonads share common primordium with adrenal glands (AGP, adrenogonadal primordium) (Fig. 1.2). In mouse, AGP forms as a thickening of coelomic epithelium as early as 9.5 *dpc*, and then by 10.5 *dpc*, the AGP splits into adrenal cortex primordium (anterior) and gonadal primordium (posterior) (Bandiera et al. 2013; Hatano et al. 1994; Ikeda et al. 1994).

The thickening of the coelomic epithelium begins at the anterior part of a mesonephros at 10.3 *dpc*. Then, the process of the genital ridge formation precedes toward the posterior half of the mesonephros. At 10.4 *dpc*, the coelomic epithelium is multilayered in the anterior part; however, the middle and posterior part of a future genital ridge is still single layered (Hu et al. 2013). Meantime, the primordial germ cells (PGCs) migrate to the genital ridges from the basis of allantois via the hindgut and mesentery. They settle down in the genital ridges between 10.0 and 11.5 *dpc* (Fig. 1.1c) (Gomperts et al. 1994; Molyneaux et al. 2001). From 11.5 *dpc*

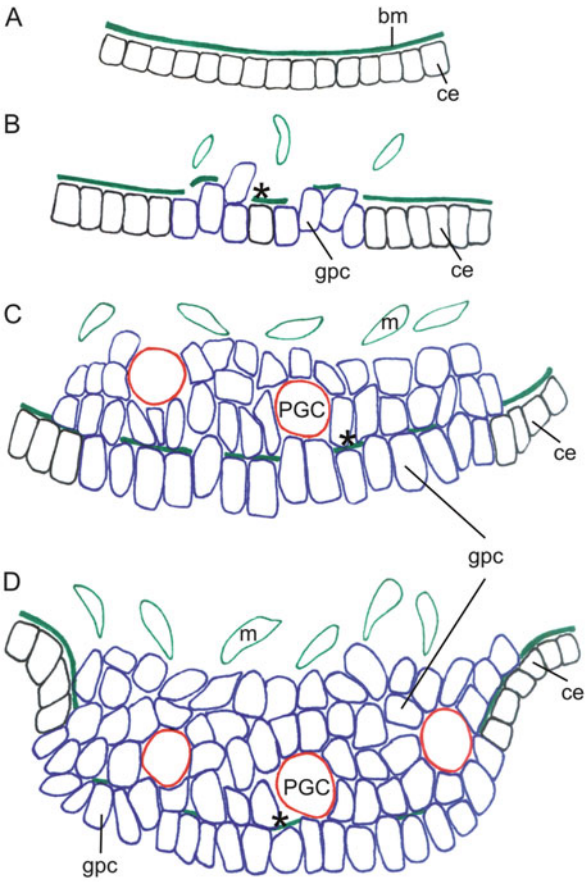

Fig. 1.1 Mouse genital ridge development. (**a**) Coelomic epithelium (ce) lined by a basement membrane (bm). (**b**) Some coelomic cells lose epithelial features, transform into SF1-positive gonadal precursor cells (gpc), and ingress through a disintegrating basement membrane. (**c**) The early genital ridge forms a cluster of coelomic epithelium-derived cells; PGCs settle among SF1-positive gonadal precursor cells; fragments of the basement membrane (*asterisk*) present near the surface of the genital ridge. (**d**) The genital ridge grows and is not covered by a true epithelial layer; mesonephros-derived cells (m) immigrate to the genital ridges

onward, the sex-specific features appear in the gonads signaling the beginning of the sexual differentiation of the gonads. In human fetus, the genital ridges form between 4.5th and fifth week of gestation and remain sexually undifferentiated by the seventh week (Francavilla et al. 1990). At the end of the fifth and during the sixth gestational week, human primitive gonads are colonized by PGCs.

1.2 Genetic Mechanisms Initiating Genital Ridge Development

The molecular control of genital ridge formation is not well understood. However, studies on mutant mice have provided key information on the genes regulating initiation of gonadogenesis. Genes participating in the regulation of the genital ridge formation were summarized in Table 1.1. It is clear that the first molecular

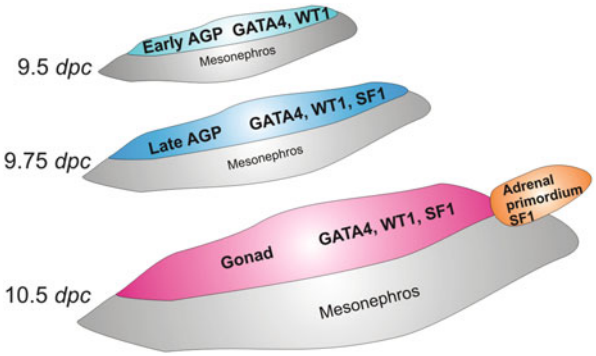

Fig. 1.2 Mouse adrenogonadal primordium (AGP) development. In early AGP at 9.5 *dpc*, the *Gata4* and *Wt1* are expressed. In late AGP at 9.75 *dpc*, *the Sf1* expression begins. At 10.5 *dpc* the gonad and adrenal primordium split; expression of *Gata4*, *Wt1*, and *Sf1* continues in the developing gonads, whereas *Sf1* is expressed in the adrenal primordium (modified from Bandiera et al. 2013)

signs of the genital ridge formation precede the earliest morphological manifestation of the genital ridge. One of the first expressed genes is *Wt1* (Wilms' tumor suppressor 1), which regulates formation of the genital ridge and kidney. Starting from 9.5 *dpc*, *Wt1* is expressed throughout the whole urogenital ridges, i.e., in the future gonads and developing kidneys. *Wt1* gene encodes 24 isoforms of zinc-finger transcription factor, among which a short isoform WT1-KTS is a key for the development of the genital ridges. Mice lacking functional WT1-KTS have impaired genital ridges due to increased cell death (Hammes et al. 2001; Kreidberg et al. 1993). *Gata4* (GATA-binding factor 4) is the earliest expressed gene specific for the genital ridge development. In mice, the expression of this zinc-finger transcription factor begins at 10.0 *dpc* (26–27 total somite stage) in the anterior half of the nascent genital ridge and then gradually extends posteriorly within the coelomic epithelium (Hu et al. 2013). In the posterior half, the expression of *Gata4* starts at 10.2 *dpc* (2 ts). The expression of *Gata4* is followed by the thickening of the coelomic epithelium in anteroposterior direction and leads to the formation of genital ridges. In mutant mouse embryos, the loss of *Gata4* expression results in single-layered and unthicken coelomic epithelium and thus in the total lack of genital ridges (Hu et al. 2013). The loss of the *Gata4* expression leads to decreased BrdU incorporation, which suggests that lack of *Gata4* abrogates proliferation of coelomic epithelium. Additionally, in *Gata4*-deficient mouse embryos, the basement membrane underlying coelomic epithelium does not disintegrate (Hu et al. 2013). In wild-type mice, the *Gata4* expression is followed by the expression of other genital ridge markers such as *Sf1* (SF1, steroidogenic factor 1, also known as Nr5a1, nuclear receptor subfamily 5, group A, member 1 *s*. Ad4BP—adrenal 4-binding protein), *Lhx9* (LIM homeobox 9), and *Emx2* (empty spiracles homeobox 2) (Birk et al. 2000; Kusaka et al. 2010; Luo et al. 1994; Miyamoto et al. 1997, 2008). These genes differ slightly in the site of their

expression. *Gata4* is expressed not only in the nascent genital ridges but also in their vicinity, i.e., in the adjacent mesenchyme, mesentery, and gut endoderm (Hu et al. 2013). *Emx2* is expressed in the coelomic epithelium at the site of genital ridge formation, adjacent mesenchyme, nephric duct, and mesonephric tubules (Kusaka et al. 2010). *Wt1* is expressed in the whole urogenital ridges (gonads and mesonephroi) from 9.5 *dpc*. *Lhx9* is expressed in the genital ridges and partially in the mesentery (Hu et al. 2013). In contrast, the expression of the orphan nuclear receptor *Sf1* is restricted to the genital ridges. Thus, the SF1 marks the identity of true gonadal somatic precursor cells. The expression of *Sf1* begins at 10.2 *dpc* (2 ts) along the anterior half of the mesonephros and then proceeds posteriorly (Hu et al. 2013). There is also a striking pattern of gene expression in the AGP, i.e., the common primordium of the gonads and adrenal gland (Fig. 1.2). In the early AGP (9.5 *dpc*), the *Wt1* and *Gata4* are expressed. In the late AGP (9.75 *dpc*), *Sf1* is expressed along with *Wt1* and *Gata4*. After AGP splits into gonadal primordium and adrenal primordium (10.5 *dpc*), the *Sf1*, *Wt1*, and *Gata4* remain expressed in the genital ridge, and only *Sf1* is expressed in the adrenal primordium (Bandiera et al. 2013). Although the timing of gene expression pattern described by Bandiera et al. (2013) differs slightly from the timing described by Hu et al. (2013), undoubtedly the differential expression of these genes is a key for development of gonads and adrenal glands from common primordium. Later in development, in sexually differentiating gonads, *Sf1* expression ceases in the coelomic epithelium covering the genital ridge but remains in the pre-Sertoli cells and fetal Leydig cells at 12.5 *dpc* and later persists only in the fetal Leydig cells (Ikeda 1996). In the developing ovaries, *Sf1* expression ends by 13.0 *dpc*.

In contrast to *Gata4* null mutants, in which the genital ridges do not form at all, in the *Wt1*, *Sf1*, *Lhx9*, and *Emx2* null mutant mice, the genital ridges start to form but degenerate about 12.5 *dpc*. The *Sf1*, *Wt1*, and *Emx2* mutants show increased cell death in genital ridges, while *Lhx9* mutants show disrupted cell proliferation (Table 1.1; Birk et al. 2000; Bland et al. 2004; Hammes et al. 2001; Kusaka et al. 2010; Luo et al. 1994; Mazaud et al. 2002). WT1-KTS (splice form lacking KTS tripeptide) is required for genital ridge formation through regulation of *Sf1* promoter in cooperation with LHX9. The expression of *Sf1* is also regulated by *Pod1* (podocyte expressed-1, basic helix-loop-helix transcription factor). POD1 represses *Sf1* expression (Cui et al. 2004; Tamura et al. 2001). *Pod1* null mutation leads to the ectopic expression of *Sf1* in gonads and mesonephroi and to the formation of hypoplastic gonads lacking the testis cords or ovarian follicles (Cui et al. 2004). Other factors involved in regulation of the genital ridge formation belong to insulin/insulin-like growth factors (IGFs). In mice lacking insulin receptor (*Insr*) and insulin-like growth factor 1 receptor (*Igf1r*), development of the gonads is impaired due to decreased *Sf1* expression and reduced proliferation of somatic cells in the genital ridges (Pitetti et al. 2013). *Emx2* null mutants also show reduced expression of *Sf1* (Kusaka et al. 2010). In *Emx2* mutants, cell migration from coelomic epithelium through the basement membrane and thus thickening of the coelomic epithelium and the formation of multilayer epithelium in the genital ridges are impaired (Kusaka et al. 2010). All these data indicate that *Gata4* is

Table 1.1 Genes participating in regulation of the early gonadogenesis and genital ridge formation

Gene symbol	Gene name	Function/role	Site and time of expression in the gonads	Loss-of-function mutation effects in mouse gonads	Loss-of-function mutation effects in human gonads	Reference
Cbx2 (*M33*)	Chromobox homolog 2	Component of the polycomb multiprotein complex	Genital ridges	Hypoplastic gonads	Gonadal dysgenesis	Katoh-Fukui et al. (2012)
Emx2	Empty spiracle homeobox 2	Homeodomain transcription factor/gonadal development	Coelomic epithelium in the site of genital ridge development, adjacent mesenchyme, nephric duct, and mesonephric tubules	Genital ridge degeneration due to the increase in cell death	Gonadal dysgenesis	Miyamoto et al. (1997); Kusaka et al. (2010)
Gata4	GATA-binding factor 4	Zinc-finger transcription factor/gonadal development	Coelomic epithelium in the nascent genital ridges from 10.0 *dpc*, adjacent mesenchyme, mesentery, gut	No genital ridges due to lack of coelomic epithelium proliferation and thickening	Normal	Hu et al. (2013); Miyamoto et al. (2008)
Insr, *Igf1r*	Insulin receptor, insulin-like growth factor 1 receptor	Receptors of insulin/IGF signaling	Coelomic epithelium descendants and germ cells	Genital ridge impaired due to decreased cell proliferation	NR	Pitetti et al. (2013)
Lhx9	LIM homeobox 9	Homeodomain transcription factor/gonadal development	Coelomic epithelium in the genital ridges and partially the mesentery	Genital ridge degeneration due to the impaired cell proliferation	NR	Birk et al. (2000); Mazaud et al. (2002)
Pod1	Podocyte expressed-1	Helix-loop-helix transcription factor	Coelomic epithelium of the genital ridge and the boundary between the gonad and mesonephros	Ectopic expression of *Sf1* and hypoplastic gonads	NR	Tamura et al. (2001); Cui et al. (2004)

Sf1 (*Nr5a1*)	Steroidogenic factor 1, nuclear receptor 5a1	Nuclear receptor/ gonad and steroidogenic gland development	Coelomic epithelium from 10.2 *dpc* and descendants	Genital ridge degeneration due to the increase in cell death	Dysgenetic or absent gonads	Luo et al. (1994); Bland et al. (2004)
Six1, *Six4*	Sine oculis homeobox homologs 1 and 4	Homeobox transcription factors	Coelomic epithelium in the nascent genital ridges from 9.5 *dpc*	Disrupted coelomic epithelial cell ingression, decreased SF1-positive gonadal progenitor cells, and decreased gonad size	NR	Fujimoto et al. (2013)
Wt1 (-KTS isoform)	Wilms' tumor factor 1	Zinc-finger transcription factor/ gonad and kidney development	Urogenital ridges from 9.5 *dpc* in mice	Genital ridge degeneration due to the increase in cell death	Dysgenetic gonads	Kreidberg et al. (1993); Hammes et al. (2001)

NR not reported

required for the initiation of genital ridge formation, whereas *Sf1*, *Wt1*, *Lhx9*, *Emx2*, and insulin/IGF signaling are necessary for the maintenance of genital ridges and their further development (Fig. 1.3a). The expression of all these genes is interdependent. For example, the expression of *Sf1* is significantly downregulated in *Gata4*-, *Wt1*-, *Lhx9*-, or *Emx2*-deficient genital ridges (Hu et al. 2013; Kusaka et al. 2010; Wilhelm and Englert 2002). In *Gata4* null mutants, *Wt1* and *Emx2* but not *Sf1* or *Lhx9* are expressed in the genital ridges (Hu et al. 2013). *Lhx9* expression depends on the *Gata4* but is not altered by the lack of *Sf1*, *Wt1*, and *Emx2* (Fig. 1.3a). The lack of *Wt1* and *Emx2* expression does not impair *Gata4* expression or the thickening of coelomic epithelium. Functional genetic experiments have revealed a complex network of interactions between the genes expressed in the primitive gonads (Fig. 1.3a). Interestingly, in mice, the set of these genes involved in the formation of the genital ridges is also responsible for the regulation of male sex-determining genes, such as *Sry* and *Sox9* (Piprek 2009a). The described above genes seem to be evolutionarily conserved among vertebrates; they are also expressed in developing gonads of zebra fish, tilapia, *Xenopus laevis*, *Trachemys scripta*, alligator, and chicken (Barske and Capel 2010; Kawano et al. 2001; Kent et al. 1995; Li et al. 2012; Oreal et al. 2002; Smith et al. 1999).

Some of the homeodomain proteins may also be engaged in the early development of gonads. These genes are key regulators of the body plan and thus may be important for spatiotemporal patterning of gonadal development. *Hoxa10*, *Hoxa9*, *Hoxa11*, and *Hoxa13* expressions have been detected in the urogenital system (Taylor et al. 1997) and thus presumably may be responsible for precise determination of the site of genital ridge formation. Mutation of *Hoxa11* affects gonadal development in mice, leading to both male and female sterility (Hsieh-Li et al. 1995). Other homeodomain proteins SIX1 and SIX4 (sine oculis homeobox homologs 1 and 4) are expressed from the onset of the genital ridge formation. Double null mutation of these genes leads to reduction in *Sf1* expression, delayed and decreased epithelial–mesenchymal transition (EMT), and disrupted coelomic epithelial cell ingression during the formation of the genital ridges, decreased SF1-positive gonadal precursor cells, and decreased gonad size (Fujimoto et al. 2013). It has been shown that SIX1 and SIX4 transactivate *Sf1* promoter (Fujimoto et al. 2013). Similarly, *Pbx1* (pre-B-cell leukemia homeobox 1) contributes to regulation of genital ridge development via upregulation of proliferation of SF1-positive cells (Schnabel et al. 2003). Mice with null mutation in *Pbx1* display decreased cell proliferation in genital ridges and highly reduced expression of *Sf1* resulting in limited expansion of SF1-positive cells and decreased gonadal growth (Schnabel et al. 2003). In addition, a polycomb protein CBX2 (chromobox homolog 2, *M33*) may play a role in regulation of HOX gene expression in the developing urogenital system. Mice lacking functional *Cbx2* show defects in gonadal development; decreased *Lhx9*, *Sf1*, and *Gata4* expression; male-to-female sex reversal; and hypoplastic gonads of both sexes (Katoh-Fukui et al. 2012). Interestingly, *Gata4*, which is the first gene expressed specifically in the genital ridges, is expressed in the coelomic epithelium located only along the mesonephros, and its expression does not extend caudally on the surface of the metanephros. It remains to be seen how the

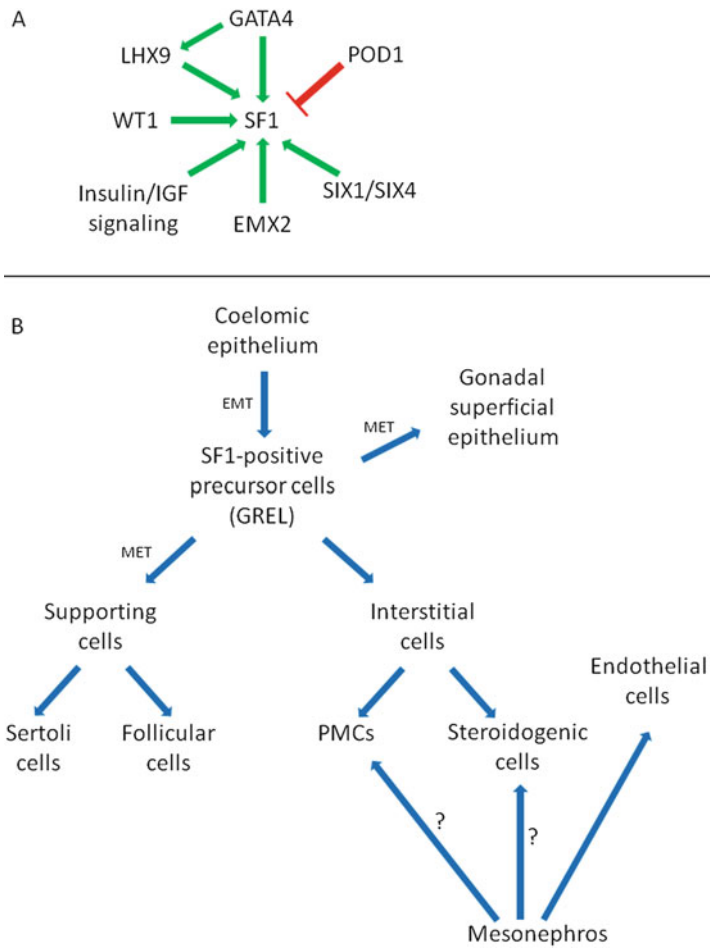

Fig. 1.3 (**a**) Interactions between genes regulating genital ridge development. *Green arrows* indicate upregulation and *red arrow* indicates downregulation. (**b**) Diagram of gonadal cell lineage origin, fate, and differentiation

genital ridge formation becomes restricted only to the site running along the mesonephros and if the mesonephros induces gonadal development.

The mentioned above genes differ not only in the spatiotemporal pattern of expression but also in their role in the genital ridge formation. It has been suggested that (1) *Gata4*, *Six1*, and *Six4* regulate formation of SF1-positive gonadal precursor cells in the coelomic epithelium; (2) *Lhx9*, *Wt1*-KTS, and IGF influence *Sf1* expression and promote genital ridge formation; (3) *Emx2* and possibly *Six1* and *Six4* contribute to EMT and cell ingression in forming genital ridges; and (4) after formation of the coelomic epithelium thickening, the *Cbx2* and *Pod1* modulate *Sf1* expression regulating cell proliferation and differentiation of the genital ridges (Tanaka and Nishinakamura 2014).

Interestingly, expression of these genes is not necessary for PGC migration and colonization. In mutants lacking *Lhx9*, *Sf1*, *Wt1*, or *Emx2* expression, the PGCs are able to colonize genital ridges. In the *Gata4* null mutants, the genital ridges do not form; nevertheless, PGCs immigrate to the monolayer coelomic epithelium at the surface of the mesonephroi (Hu et al. 2013). This shows that the presence of genital ridges is not required for the PGC migration, guidance, or settlement. It has been postulated that developing gonads secrete SDF1 factor that attracts PGCs (Doitsidou et al. 2002; Molyneaux et al. 2003). However, it must be emphasized that in *Gata4* null embryos, the SDF1 continues to be secreted by the mesentery and mesonephros starting from 9.0 *dpc*, and probably because of this, the PGCs can still migrate to the site of presumed genital ridges. On the other hand, the PGCs are not required for the initiation or development of the genital ridges (McLaren 1991).

1.3 Cell Proliferation in Early Gonadal Development

The gonads' development depends not only on cell proliferation but also on cell migration from the adjacent mesonephric mesenchyme. The primordial gonads initially derive from, at least, three cell lines: coelomic epithelium-derived cells, mesonephros-derived cells, and germ cells. These three cell lines give rise to a multitude of cell types in adult testes and ovaries. In early mouse gonads, the most intensive proliferation has been described for coelomic epithelium-derived cells, which cover genital ridges (Schmahl et al. 2000; Schmahl and Capel 2003). In addition, the most robust cell divisions have been reported for the XY gonads (genetic males) indicating the importance of cell proliferation for early stages of testis development. There are two phases of male-specific proliferation in developing gonads. The earliest phase of proliferation affects SF1-positive cells in coelomic epithelium that will give rise to the precursors of Sertoli cells (pre-Sertoli cells) and possibly also other cell lineages (Fig. 1.3b). This phase takes place between 11.0 and 11.5 *dpc* and is promoted by fibroblast growth factor 9 (FGF9) acting via FGFR2 (Karl and Capel 1998; Piprek 2010; Schmahl et al. 2000). *Fgfr2* null mutants show decreased proliferation of somatic cells in XY gonads and impaired differentiation of Sertoli cells (Kim et al. 2007; Piprek 2010). It is not clear if Sertoli cells originate from SF1-positive gonadal precursor cells preexisting in the genital ridges or from cells that ingress inward the genital ridges at later stages. Lineage tracing indicated that Sertoli cells in mice originate from cells that enter the interior of the genital ridges from the superficial layer within 2-h window of time between 11.2 and 11.4 *dpc* (Karl and Capel 1998; Schmahl et al. 2000). After *Sf1* expression in the coelomic epithelium ceases, the precursors of Sertoli cells stop dividing, but SF1-negative cells of coelomic epithelium continue to proliferate. Cells originating in this later phase of coelomic epithelium proliferation (11.4–12.5 *dpc*) give rise to interstitial cells only. This later phase of male-specific proliferation is promoted by PDGF rather than by FGF9, since the loss of *Pdgfr*α receptor disrupts only this latter phase of proliferation and does not influence the early proliferation of

SF1-positive cells (Brennan et al. 2003; Piprek 2010). As a result of enhanced proliferation of somatic cells in the male gonads, the differentiating testis becomes almost twice the size of the ovary. Proliferation in developing ovaries is not as robust as in the male gonads, and additionally the follicular cells probably originate from coelomic epithelium-derived cells ingressing at the earliest stages of gonadogenesis, which give rise to FOXL2-positive granulosa of medullary follicles, but also during later development (around birth) from LGR5-positive cells giving rise to the granulosa of cortical follicles (Mork et al. 2012).

1.4 Cellular Events in Genital Ridge Formation in Mice and Other Vertebrates

The ventral surface of the urogenital ridges is covered by coelomic epithelium (monolayer of cuboidal cells in the mouse or flat cells in the frog *X. laevis*) and underlined by a thick basement membrane separating the epithelium from the mesonephric mesenchyme and nephrons (Fig. 1.1). As it was mentioned above, the first cellular sign of genital ridge formation is disintegration of the basement membrane under coelomic epithelium at the site of gonadal development. In mouse, the fragments of disintegrating the basement membrane are visible beneath the epithelium-like layer at the surface of genital ridges (Karl and Capel 1998). Disintegration of the basement membrane under the epithelium covering the genital ridges was also observed in human, prosimian *Galago*, swine, and bovine (Hummitzsch et al. 2013; Pelliniemi et al. 1998; Pereda et al. 2001; Satoh 1991; Yoshinaga et al. 1988). The disintegration of the basement membrane occurs through the action of extracellular matrix (ECM) digesting enzymes such as metalloproteinases. It would be interesting to study the ECM-remodeling enzymes during the formation of the genital ridge and to test how these enzymes influence early gonadogenesis. Disintegration of the basement membrane allows the proliferating cells of the coelomic epithelium to ingress and form a cluster of SF1-positive gonadal somatic precursor cells constituting the genital ridge (Fig. 1.1). In bovine embryos these coelomic-derived gonadal somatic precursor cells have been named the GREL cells (genital ridge epithelial-like cells) (Hummitzsch et al. 2013). In summary, during the primordial gonad formation, the coelomic epithelial cells of the genital ridges proliferate, partially lose their epithelial features, and transform into gonadal precursor cells (GREL cells) and thus undergo EMT transition and move inward (Hummitzsch et al. 2013; Karl and Capel 1998; Schmahl and Capel 2003). It is still unknown if these superficial cells actively migrate or passively ingress due to the high rate of proliferation. The enhanced divisions of superficial cells (indicated by high incorporation of BrdU) of the genital ridges lead to the growth of gonadal primordium (Schmahl and Capel 2003). Superficial cell labeling of the genital ridges with fluorescent lipophilic dye DiL in mice or with a mixture of MitoTracker and rhodamine derivative in a

red-eared slider turtle *Trachemys scripta* showed the ingression of coelomic epithelium-derived cells inward into the gonadal primordium (Karl and Capel 1998; Yao et al. 2004). This proved the origin of the first gonadal cells from the coelomic epithelium. The onset of gonadogenesis, which is marked by the formation of coelomic epithelium thickening, is similar in mouse, bovine, chicken, and *T. scripta* and is probably characteristic for all amniotes. However, *T. scripta* and chicken early gonadogenesis is an exception due to the lack of basement membrane disintegration under proliferating epithelium. Thus, in these species the increase in cell number does not lead to the formation of an unorganized cell mass but to the formation of the fingerlike sex cords covered by the basement membrane (Fig. 1.4). The genital ridge formation is slightly different in lower vertebrates such as *X. laevis*. Here the first sign of gonadal development occurs when a monolayer coelomic epithelium composed of flat cells bulges into the coelomic cavity forming mounds containing PGCs (Wylie and Heasman 1976; Fig. 1.5). Interestingly, in amphibians the coelomic epithelium does not form thickenings at the onset of genital ridge development, which is characteristic for amniotes.

Studies of the early gonadal development have been facilitated by identification of molecular markers. Several molecular markers have been identified following the microarray analysis of gene expression in the gonadal precursor cells (GREL cells) of bovine embryos (Hummitzsch et al. 2013). GREL cells differentiating from typical epithelial cells of gonadal surface still express some epithelial-specific markers such as cytokeratins 18 and 19, plakophilin 2, and desmoglein 2, but also express StAR, which marks the steroidogenic cells. However, GREL cells do not express genes specific for gonadal surface cells such as adipophilin, fibulin 2, merocin, or mucin 1. This implies that GREL cells are intermediate cell type between the coelomic epithelium cells and the mesenchymal cells.

Early studies indicated that the developing gonad is covered by a superficial epithelium, which proliferates and gives rise to the somatic cells of the gonad (Karl and Capel 1998), but later studies showed that developing gonad is not covered by a true epithelium. A definitive epithelium is always composed of a layer of polarized cells lying on a basement membrane; however, the genital ridge is a mass of cells and is not organized into the cell layer (Figs. 1.1 and 1.6). This was clearly showed in the studies on early gonadogenesis in bovine embryo (Hummitzsch et al. 2013). The gonadal hilum (basal region) is the only region of the primitive gonad covered by a true epithelium and is continuous with the mesonephric superficial epithelium. This region of gonadal epithelium is a source of the LGR5-positive stem cells that are also present in adult ovaries and play an important role in ovarian surface regeneration after ovulation (Flesken-Nikitin et al. 2013).

The early genital ridge is built of a cluster of somatic cells among which the primordial germ cells settle (Fig. 1.1). Later, the stromal cells migrate from the mesonephric mesenchyme into the developing genital ridge (Figs. 1.1 and 1.6) (Capel et al. 1999). Consequently, the primordial gonads are composed of cells of various origins. The stromal (mesenchymal-like) cells derived from mesonephroi invade the genital ridges, which in mice occurs at 11.5 *dpc* and in bovine between 70 and 130 days of gestation (Hummitzsch et al. 2013; Tilmann and Capel 1999).

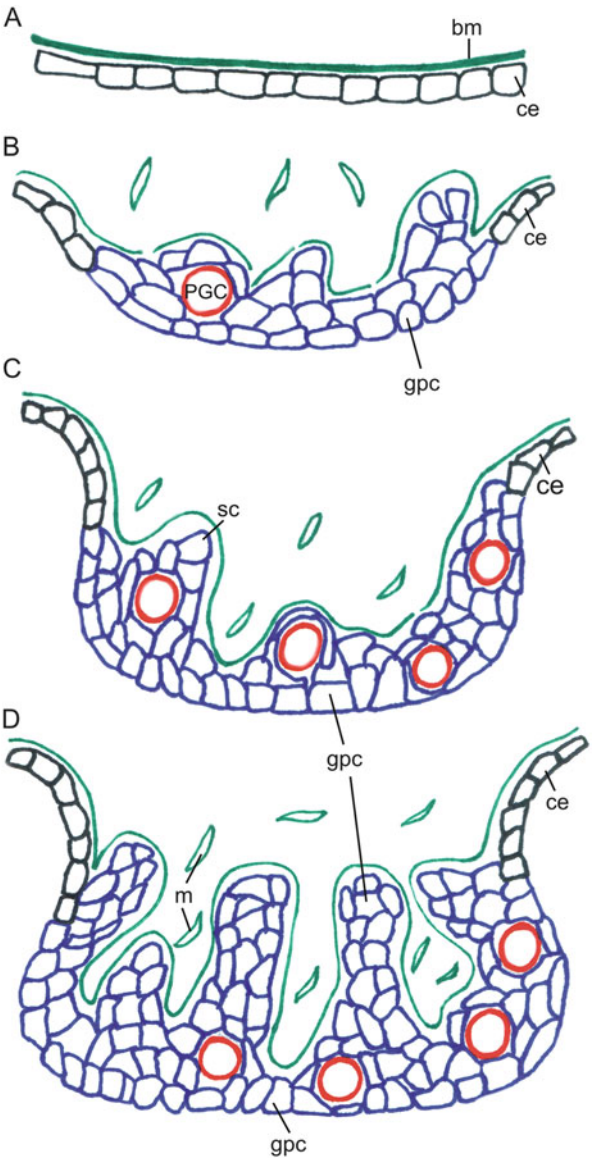

Fig. 1.4 Diagram of genital ridge development in the turtle *Trachemys scripta* and chicken. (**a**) Coelomic epithelium (ce) lined by the basement membrane (bm). (**b**) Coelomic epithelial cells transform into SF1-positive gonadal precursor cells (gpc or GREL cells) and ingress forming fingerlike cords covered by continuous basement membrane. (**c**) Proliferation of the gonadal precursor cells leads to the growth of primitive sex cords (sc) that contain primordial germ cells (PGCs). (**d**) Sex cords (sc) grow; in the undifferentiated gonads, the germ cells are located in the peripheral, cortical region. Mesonephros-derived cells (m) invade the genital ridges and locate between the sex cords

The mesonephric cell migration was also observed in amphibians but not in the turtle *T. scripta* (Piprek et al. 2010; Yao et al. 2004). In bovine embryo the stromal cells migrating from the mesonephroi have a mesenchymal character and are surrounded by extracellular matrix containing collagen type I, fibrillin 1, fibronectin, and decorin. In contrast, the extracellular matrix of GREL cells consists of collagen types IV and XVIII, laminin, perlecan, and nidogen (Hummitzsch et al. 2013). Both the coelomic epithelium-derived and mesonephros-derived

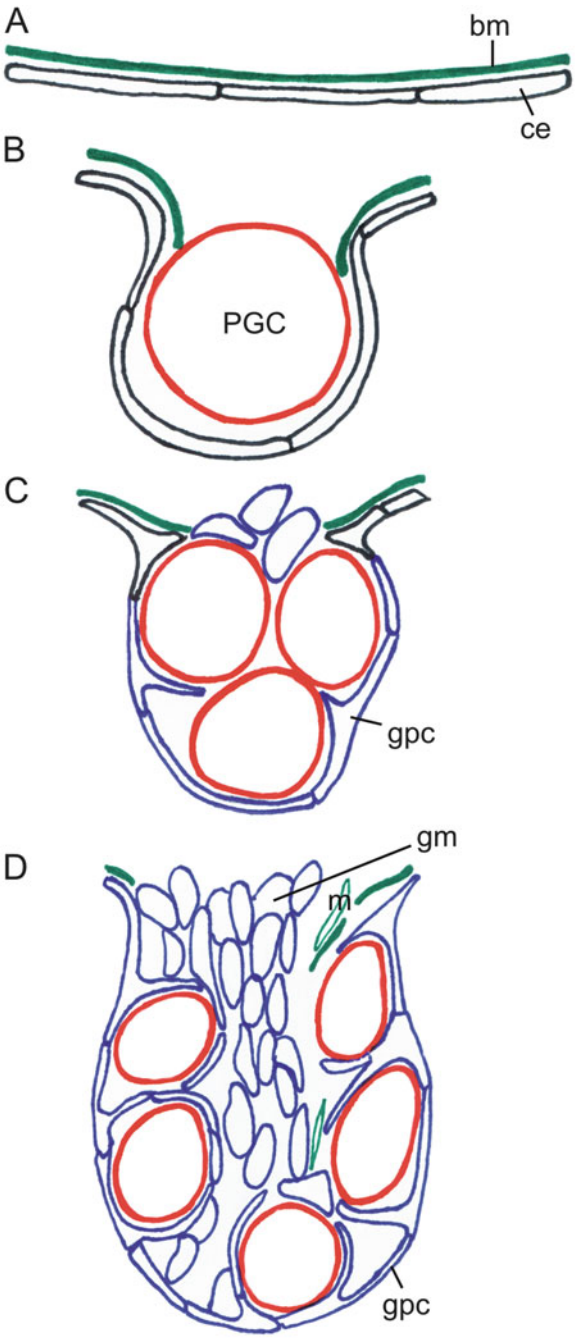

Fig. 1.5 Diagram of genital ridge development in the African clawed frog *Xenopus laevis*. (**a**) Coelomic epithelium (ce) lined by the basement membrane (bm). (**b**) PGC settlement results in formation of the genital ridges that bulge to the coelomic cavity. (**c**) Coelomic epithelial cells transform into gonadal precursor cells (gpc) that proliferate, ingress, and enclose the PGCs. (**d**) Coelomic epithelium-derived cells locate in the center of the genital ridges forming the gonadal medulla (gm). Mesonephros-derived stromal cells (m) ingress between the medulla and superficial region (cortex)

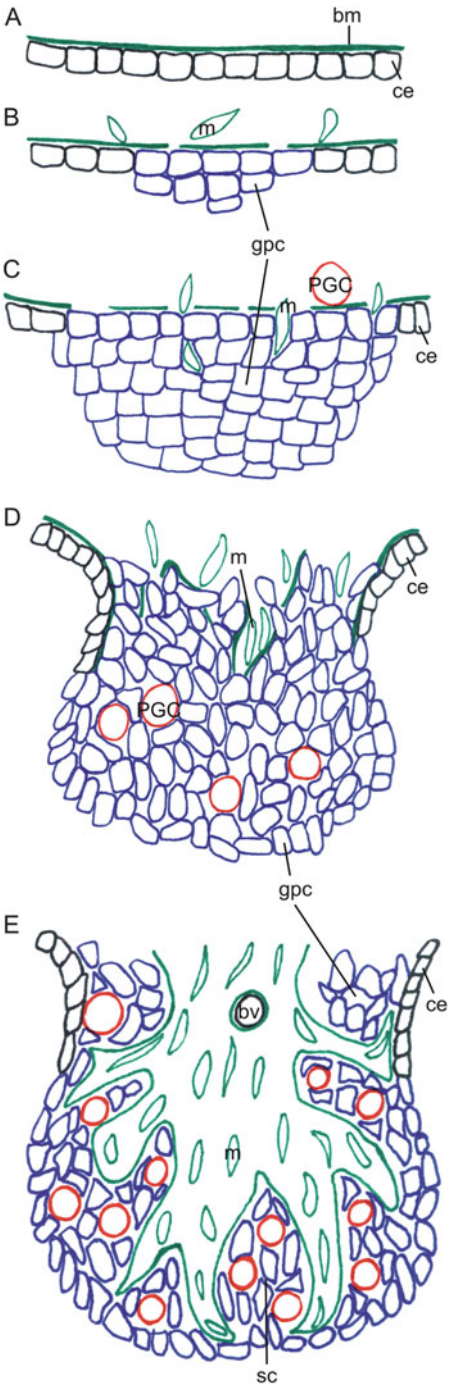

Fig. 1.6 Diagram of genital ridge development in the bovine embryo. (**a**) Coelomic epithelium (ce) lined by the basement membrane (bm). (**b**) Coelomic epithelial cells transform into SF1-positive gonadal precursor cells (gpc) also termed GREL cells that proliferate and form a cluster; the basement membrane disintegrates. (**c**, **d**) PGCs and mesonephros-derived cells (m) invade the genital ridges. (**e**) The basement membrane forms at the interface between the mesonephros-derived and GREL cells, and the sex cords (sc) develop; blood vessels (bv) form in the stromal region (modified from Hummitzsch et al. 2013)

stromal cells produce ECM in the primitive gonads. The basement membrane forms at the interface between the GREL and stromal cells, which contributes to the establishment of gonadal structure. The stromal cells penetrate the gonads toward their surface separating the sex cords. Migrating stromal cells reach the space under the surface of the gonads and induce differentiation of the superficial cells into the true epithelium covering the gonads.

The formation of sex cords is a first sign of compartmentalization in developing gonads. Within the sex cords, the coelomic epithelium-derived gonadal precursor cells (GREL cells) enclose germ cells, and afterward they differentiate into Sertoli cells in the developing testes or follicular (granulosa) cells in the developing ovaries (Fig. 1.6). In mice, the sex cords do not form in the undifferentiated gonads; they form only in the differentiating testes. The formation of testis cords depends on the cell migration from the mesonephros (Combes et al. 2009). In bovine, the sex cords (testis and ovigerous cords) develop in XX and XY gonads when the mesonephros-derived cells invade the gonads. Subsequently, the basement membranes form at the interface of GREL and stromal cells. This indicates that the mesonephros-derived cells and timing of their migration are the keys for the organization of gonadal structure. As it was mentioned before, in *T. scripta* and chicken, the sex cords appear earlier in development when the cells of thickened coelomic epithelium proliferate, which leads to the growth of the fingerlike primitive sex cords protruding inward the genital ridges from the thickened coelomic epithelium (Smith and Sinclair 2004; Yao et al. 2004) (Fig. 1.4). In these species the sex cords are covered by the basement membrane that is continuous with the basal membrane of the coelomic epithelium.

In amphibians, the structure of the primitive gonads is quite unique; the sex cords are not present, the germ cells remain in the peripheral (cortical) position in connection with the superficial cells of the gonads, and the proliferating somatic cells gather in the center of the primitive gonad forming a sterile medulla (Fig. 1.5). The medullary cells originate from the coelomic epithelium (Falconi et al. 2004; Iwasawa and Yamaguchi 1984; Merchant-Larios 1979; Merchant-Larios and Villalpando 1981; Piprek et al. 2010; Tanimura and Iwasawa 1988, 1989). Later in development, mesenchymal cells immigrate from the mesonephroi into the gonads and locate between the gonadal cortex and medulla. Eventually the basement membrane forms at the interface of the coelomic epithelium-derived cells and the stromal cells (Fig. 1.5).

The fate of the bipotential gonads is established in the sex determination process that occurs in sexually undifferentiated gonads. The molecular mechanism determining sexual differentiation of the gonads has been described in Chap. 3 of this volume. In mice, in XY gonads the SF1-positive gonadal precursor cells derived from the coelomic epithelium start expressing *Sry* gene followed by the expression of *Sox9* gene (Piprek 2009a). These two genes determine the male sex in mammals and trigger the differentiation of gonadal precursor cells into Sertoli cells that enclose the germ cells and gather into the testis cords, which later in development give rise to the seminiferous tubules (Combes et al. 2009). In XX murine, gonads *Rspo1* and *Wnt4* are upregulated; here, the SF1-positive gonadal precursor cells

gather into the ovarian follicles in which they enclose (as a follicular or granulosa cells) a single oocyte (Piprek 2009b; Yao et al. 2004). Thus, Sertoli and follicular cells share common origin (Albrecht and Eicher 2001). It seems that the ingressing coelomic epithelial cells first undergo EMT (they lose epithelial features to form a cluster of somatic cells inside the genital ridges) and then some of these cells commit to MET (mesenchymal–epithelial transition) when they regain epithelial features differentiating into supporting cells. Nevertheless, gonadal precursor cells do not have typical mesenchymal character; and they are rather an intermediate stage between epithelial and mesenchymal cells. In bovine, unlike in mice, both in XX and XY gonads, the somatic gonadal precursor (GREL) cells initially gather into sex cords (Hummitzsch et al. 2013). In XY bovine gonads, GREL cells in the cords differentiate into the Sertoli cells, and the cords develop into seminiferous tubules; however, in XX gonads, GREL cells give rise to follicular cells and the sex cords are fragmented into ovarian follicles.

Interestingly, the supporting cells are not the only cells derived from coelomic epithelium in the primitive gonads. Cell-tracking experiment revealed that the SF1-positive gonadal precursor cells are also present between the sex cords and probably give rise to the interstitial lineages (Fig. 1.3b) (Karl and Capel 1998). Figure 1.3b depicts origin and differentiation of gonadal cell lineages. Originally, it has been postulated that the interstitial cells (including steroidogenic cells) originate from the stromal cells migrating from the mesonephros (Buehr et al. 1993; Martineau et al. 1997; Merchant-Larios et al. 1993). Studies on mouse testis development showed that the mesonephros-derived cells give rise mainly to the endothelial cells and thus the gonadal vasculature; however, the interstitial cells probably originate from the coelomic epithelium (Combes et al. 2009; Cool et al. 2008). It has been shown that two interstitial cell lineages, the peritubular myoid cells (PMCs) and the fetal Leydig cells (FLCs), and possibly also the adult Leydig cells (ALCs) originate from the SF1-positive coelomic epithelium-derived cells (Barsoum et al. 2013; Brennan et al. 2003; Combes et al. 2009; Cool et al. 2008). Importantly, Liu et al. (2015) showed that steroidogenic cells in developing mouse ovaries have a dual origin: coelomic epithelial and mesonephric. It seems that some of the theca cells originate from WT1-positive cells migrating from the coelomic epithelium, and others differentiate from Gli1-positive mesenchymal cells migrating from the mesonephros (Liu et al. 2015). This may indicate that the coelomic epithelium-derived and mesonephros-derived cells form a pool of mixed multipotent precursor cells that randomly commit to different cell fates in the genital ridges. However, it is more probable that the genital ridge is not an unorganized mass of multipotent cells that will differentiate into various gonadal cell lines but that the fate of gonadal cells is determined early: for example, cells originating from the first wave of coelomic epithelium proliferation give rise to the supporting cells, whereas later wave of coelomic epithelium proliferation gives rise to steroidogenic cells. Thus, timing of cell origin may determine the fate of the cells. The origin of gonadal cell fates and the mechanisms of their differentiation still require further studies.

1.5 Conclusion

The analysis of early gonadogenesis reveals a sequence of processes leading to the formation of the bipotential gonad anlage, which differentiates into the ovaries or testes. The first step of gonadal development determines the site of genital ridge formation. Although the molecular mechanism of this process is still obscure, studies of mutant mice have pointed to a group of genes involved in this earliest step of gonadal development. At the site of gonadogenesis, the cells of the coelomic epithelium of the ventromedial surface of mesonephroi undergo transformation; they proliferate, the underlying basement membrane disintegrates, and these result in the formation of genital ridges. The basement membrane disintegration depends on activation of ECM-remodeling enzymes such as metalloproteinases. The proliferating cells leave the coelomic epithelium, lose their epithelial features, undergo EMT, and invade the interior of the primitive gonad either individually (mice, frogs) or in the form of cords (reptiles, birds). The cells in the site of gonadogenesis secrete chemoattractants, which guide migrating PGCs toward the genital ridges. PGCs settle and associate with the cells of the thickened coelomic epithelium. The genital ridges are not required for PGC immigration to the site of gonadogenesis, and PGCs are not needed for genital ridge formation. In the genital ridges, the cells of the coelomic epithelium transform into a cluster of SF1-positive cells (termed GREL cells in bovine embryos), and thus the genital ridges are not covered by true epithelium. In all studied vertebrates, the cells derived from the coelomic epithelium give rise to the supporting cells and presumably also to other cell types, such as Leydig and theca cells. The cells of the developing gonads segregate and the gonad compartmentalizes. In amphibians, distinct cortex and medulla appear in the developing gonad. In reptiles and birds, the thickened coelomic epithelium form the cortex from which the sex cords protrudes constituting the medullary region of the gonad in both sexes. In mice, the testis cords and ovarian follicles emerge from the cell cluster just after the sex determination period. Importantly, the gonads originate not only from the cells deriving from the coelomic epithelium but also from the mesonephric mesenchyme, and a specific sequence of changes is cell proliferation, cell adhesion, cell movement, aggregation, deposition of the basement membrane, and cell differentiation that lead to the formation of the testes or ovaries.

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