CHAPTER

4

Embryonic Sertoli Cell Differentiation

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I. INTRODUCTION

In the past 10 years, we have experienced a veritable explosion in the amount of information that has been uncovered on genes important in the regulation of testis differentiation. With the identification of the testis determining gene, Sry, and localization of its expression in the Sertoli cell, new information has been elucidated on factors regulating the male sex differentiation pathway. The Sertoli cell is the critical cell type that initiates development of testis-specific gene expression, induces testis morphology, and establishes crucial parameters for spermatogenic function and capacity. Therefore, proper differentiation of the Sertoli cell during embryonic testis differentiation is mandatory for normal adult testis development and function. In this chapter, we examine embryonic testis development and the crucial role of the Sertoli cell in both sex determination and morphological events that result in the formation of a testis. The events regulating testis development are discussed using primarily the mouse as a model; however, comparisons will be made to rats, domestic livestock, and humans where information is available.

II. OVERVIEW OF EMBRYONIC TESTIS DEVELOPMENT

The genital ridge is primarily composed of a single layer of coelomic epithelium from 9 to 9.5 days postcoitus (dpc) in the mouse (Fig. 4.1) [1]. At 9–10 dpc the primordial germ cells (PGCs) migrate from extragonadal sites within the yolk sac to colonize the urogenital ridge [2, 3]. The gonad is bipotential after germ cell migration and morphologically can be distinguished from the adjoining mesonephric tissue but cannot be identified as an ovary or a testis [4].

Two morphological events occur to alter the bipotential gonad at 11 dpc of development. First, Sertoli cells differentiate in part from the coelomic epithelium and start to proliferate, aggregating with the PGCs. Second, mesonephric cells (endothelial or preperitubular in origin) migrate from the mesonephros and enclose the pre–Sertoli-PGC aggregates to form seminiferous cords (Fig. 4.1) [5]. Both of these events rely on expression of Sry by the developing Sertoli cell [6]. As the Sertoli cell differentiates (11–13 dpc) it acquires the ability to produce Müllerian inhibiting substance (MIS), which inhibits Müllerian duct growth. The Müllerian duct is the precursor female reproductive tract that differentiates into the cervix, uterus, oviduct, and portions of the anterior vagina [7, 8].

After seminiferous cord formation, cells within the interstitium differentiate to form immature Leydig cells, while the preperitubular cells further differentiate to form a single layer of cells enclosing each seminiferous cord. In the rat, steroidogenesis occurs within

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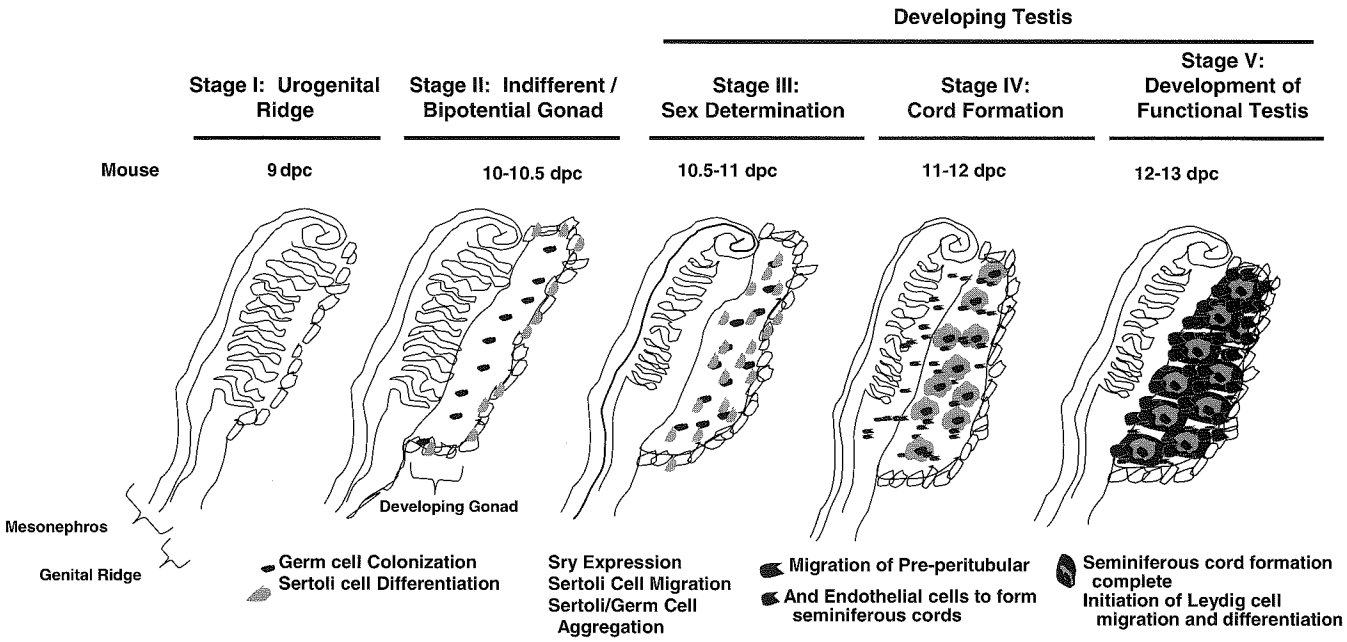

FIGURE 4.1 Morphological stages that occur during testis differentiation from genital ridge formation to cord formation and cell proliferation in the mouse with staging in number of tail somites (ts) and days postcoitus (dpc).

the testis at 14.5 dpc when 3β-hydroxysteroid dehydrogenase (3β-HSD) is first expressed in pre-Leydig cells [9, 10]. Testosterone produced by the Leydig cells maintains and stabilizes the Wolffian duct, which is the precursor of the male reproductive tract structures: the epididymis, vas deferens, and portions of the secondary sex glands [11]. The seminiferous cords develop lumen around puberty to become the seminiferous tubules. A major function of the Sertoli cell within the testis is to provide the appropriate environment (e.g., production of proteins and growth factors) and cytoarchitectural support for the developing germ cells [12]. The comparisons between events that occur in the mouse, rat, and pig during testis development and correlated time points in embryonic testis development are presented in Table 4.1.

III. STAGES AND TIMING

There are at least five different morphological stages of testis development, which are depicted in Fig. 4.1: (1) development of a genital ridge, (2) formation of an indifferent or bipotential gonad, (3) sex determination, (4) induction of testicular cords in the testis, and (5) development of a functional testis. These stages are represented in Fig. 4.1 by timelines in the mouse and are compared to other species (human, pig, cattle, sheep, and rats) in Table 4.1.

The first two stages, genital ridge formation and formation of an indifferent gonad, occur whether the individual has XX or XY chromosomes and, thus, is independent of testis or ovarian development. The last three stages of gonadal development are dependent on genes that are expressed within the indifferent gonad to result in either a testis or ovary. Expression of the gene Sry (sex-determining region of the Y chromosome) by the Sertoli cell directs the indifferent gonad to become a testis [13–15], whereas the absence of Sry and expression of Wnt4 appear to regulate formation of an ovary [16]. Thus, formation of testis-specific morphogenic structures such as seminiferous cords is dependent on differentiation of the Sertoli cell, expression of Sertoli cell–specific genes, and proliferation of the Sertoli cell population to result in normal testis development (reviews [1, 17]).

A. Stage I: Genital Ridge Development

The initial step in the development of gonads is the formation of the genital ridge (Fig. 4.1) and urogenital system from the intermediate mesoderm; this step begins at 9–9.5 dpc in the mouse. The Wolffian duct, which is the precursor of the male reproductive tract system, is derived from lateral mesoderm and runs the length of the urogenital system and develops from the mesonephric duct [18]. The Müllerian duct, which is the precursor of the female reproductive system, appears between 11.5 and 12.5 dpc from invaginations

TABLE 4.1 Gestational Age at Each Stage of Testis Development in Humans, Pig, Cattle, Mice, Rats, and Sheep

Species	Genital ridge	Bipotential gonad	Testis cord formation	Reference
Human				
Gestational age	5 weeks	6 weeks	7–8 weeks	Sinisi *et al.*, 2003
Pig				
Gestational age	18–20 days	21 days	26 days	McCoard *et al.*, 2001
Crown rump length	8–10 mm	10–12 mm	15 mm	
Cattle				
Gestational age	27–31 days	32–39 days	40 days	Wrobel and Sub, 1998
Crown rump length	<11 mm	11–18 mm	>19 mm	
Mouse				
Gestational age	9–10 dpc	10–11.5 dpc	12.5 dpc	Karl and Capel, 1998
Total no. of somites	13–28 s	29–48 s	49–52 s	Rugh, 1968
No. of tail somites	<9 s	9–18 s	24–30 s	
Rat				
Gestational age	10–11 dpc	11.5–12.5 dpc	13.5–14 dpc	Magre and Jost, 1984; Magre *et al.*, 1980; Magre *et al.*, 1981
Sheep				
Gestational age	20	23 dpc	30–35 dpc	Pellinieme *et al.*, 1981; Payen *et al.*, 1996; Quirke *et al.*, 2001; Mauleon, 1961; McNatty *et al.*, 1995

of surface epithelium from the mesonephros running parallel to the Wolffian duct [1, 17]. Only one of the two ductal systems, Müllerian or Wolffian, will develop further in mammals. The duct that develops is totally dependent on gonadal differentiation (whether an ovary or testis develops) and expression of factors that will support or regress the female or male ductal structures [11, 19]. The Sertoli cell of the testis will produce MIS, which will cause regression of the precursor female reproductive structures, whereas Leydig cells, which differentiate much later in testis development, will produce testosterone, allowing for maintenance of the male reproductive tract structures (Wolffian duct [11]).

Histologically, the formation of the genital ridge is the formation of a pair of mounds on either side of the dorsal mesentery of the hind gut at 9 dpc in the mouse. These "mounds" are formed by a single layer of coelomic epithelial cells that almost immediately begin to proliferate and thicken (Fig. 4.1) [4]. In the pig, the genital ridge forms around embryonic day 20–21 of the approximate 114-day gestation and, like the rodent, consists of a single layer of coelomic epithelium that presumably migrates from the dorsal mesentery to line the mesenchymal layer forming the genital ridge [20, 21] (Table 4.1).

1. Gene Expression Resulting in Proliferation of the Genital Ridge and Indifferent Gonad Formation

Several genes encoding transcription factors mediate the early events in the development of the indifferent gonads. Lhx1 (Lim 1), Wilms' tumor 1 (Wt1), steroidogenic factor 1 (Sf1), Emx2, and Lhx9 are all expressed in the urogenital ridge by 9.5 dpc (Fig. 4.2). Mice with disruptions in any of these genes have arrested gonadal development, and the mice die shortly after birth from developmental defects in the adrenal cortex (Sf1), kidneys (Wt1, Emx2), or kidneys and brain (Lhx1) [22–27]. Of these genes, the null mutants of Sf1, Wt1, and Emx2 all have similar phenotypes because the gonad starts to form and then it regresses. Thus, these genes are thought to be critical in expansion of the initial genital ridge.

Lim1 or *Lhx1* is the gene that has the least defined function of all the genes expressed at this time in

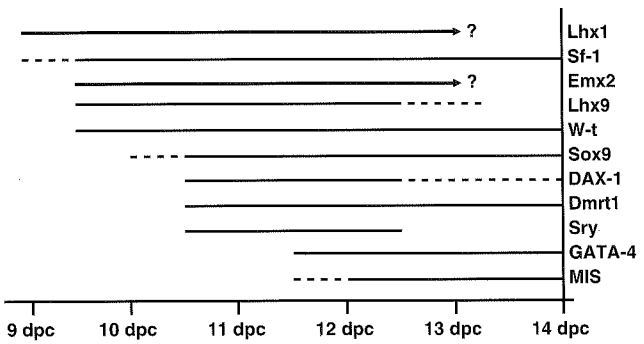

FIGURE 4.2 Expression of Sertoli cell genes during mouse testis development. References for each gene are as follows: Lhx1: [22], Sf1: [1, 141], Emx2: [25], Lhx9: [27], Wt1: [1, 30, 35], Sox9: [1, 141], DAX-1: [1, 94, 95, 141], Dmrt1: [89], Sry: [1, 141], GATA-4: [141], and MIS [141].

gonadal development. Lim1 is a homeodomain protein that is expressed throughout the mesonephric ductal system and its expression is detected by 9 dpc (Fig. 4.2). Lim1 mutants that survive do not have kidneys or gonads; however, the exact stage of gonadal disruption has not yet been determined [22].

The Wilms' tumor suppressor gene *Wt1* was initially identified as a gene inactivated in a subset of Wilms' tumors, a form of pediatric kidney cancer [28, 29]. Inactivation of both Wt1 alleles results in embryonic lethality and a failure of kidney and gonadal development [23]. Wt1 expression occurs in the genital ridge of the indifferent gonad around 9.5 dpc (Fig. 4.2) and then becomes localized to the Sertoli cells of the testis and granulosa cells of the ovary [30, 31]. An initial function of the Wt1 gene is in the formation of a subset of mesonephric tubules, but its role in early gonadal development is not known [32]. In Wt1 mutant embryos, a thickening of the coelomic epithelium occurs, but there is no further development of the gonad and by 14.5 dpc the gonad is completely regressed [23]. Thus, a potential role for Wt1 in the gonad is in proliferation and thickening of the coelomic epithelium within the genital ridge to become the indifferent gonad.

In humans, a number of additional diseases were shown to be associated with Wt1 mutations that primarily resulted in urogenital abnormalities [33]: WAGR (Wilms' tumor, aniridia, genitourinary abnormalities, mental retardation), Denys-Drash (DDS), and the Frasier syndrome. The major abnormalities associated with these diseases are varied and can range from cryptorchidism and hypospadia to streak gonads and sex reversal of internal and external genitalia [34–38]. The absence of expression of Wt1 does not affect Sox9 or Lhx9 expression; however, both DAX-1 and Sf1 were determined to be absent in Wt1 mutant mice when compared to wild-type controls [39]. Furthermore, Wt1 also has been determined to activate the endogenous promoter of DAX-1 [40]. Therefore, both Sf1 and DAX-1 expression may be regulated directly or indirectly by Wt1.

The *Sf1* gene encodes an orphan nuclear receptor that regulates the expression of several genes involved in gonadal development and steroidogenesis [41]. In the mouse, targeted deletions of both Sf1 alleles result in the absence of both the adrenal gland and the gonad, supporting a role for Sf1 as an essential regulator of the first endocrine cells in the gonad [24]. In the early gonad, Sf1 mRNA can be detected at 9–9.5 dpc (Fig. 4.2) and is localized to the coelomic epithelium and the mesenchymal cells of the mesonephros between the tubules. The gonads of Sf1 mutant embryos do not develop beyond the early indifferent stage and XY Sf1 mutant embryos display sex reversal [24]: The Müllerian ducts develop into uteri, oviducts, and upper vagina.

Sf1 disrupted mice lack both adrenal glands and gonads supporting the suggestion that Sf1 is an essential regulator of the first endocrine cells within the gonad. Ftz-fl gene disrupted mice (encodes both Sf1 and the alternative transcript ELP) are normal at 10.5 dpc; however, at 11.5 to 12 dpc a number of the cells on the gonads degenerate via apoptosis or programmed cell death. Wt1 expression is normal in both Sf1 and Ftz-fl mutant embryos, which supports previous research from Wt1 mutants with no Sf1 expression that Wt1 is critical in inducing expression of Sf1. In Lhx9 mutants only minimal expression of Sf1 was detected [27], but normal expression of Wt1 occurred. These results suggest that Wt1 is necessary but not sufficient to activate Sf1 expression. Recent research has also determined that Lhx9 can bind to the Sf1 promoter and induce expression of Sf1 [39]. It appears that Wt1 and Lhx9 bind to Sf1 and have an additive effect on induction of expression of Sf1 in the early gonad [39].

Expression of *Lhx9* occurs in the genital ridges of mice around 9.5 dpc (Fig. 4.2) and by 11.5 it is expressed at high levels in the cells of the coelomic epithelium, whereas more moderate levels are expressed in the adjacent mesenchyme (future tunica albuginea [27]). At E13.5 (after morphological testis development), expression of Lhx9 is in the interstitial mesenchyme and less within the testicular cords. Thus, as morphological testis differentiation is completed, the expression of Lhx9 is downregulated in the cells of the coelomic epithelium. At 11.5 dpc there is no difference in the gonadal structures between Lhx9 gene–deficient mice and wild-type mice. However, by 12 dpc no proliferation was evident in the Lhx9 mutants and by 13.5 dpc no discrete gonads had formed in the Lhx9-deficient embryos as seen in the wild-type mice. Lhx9-deficient embryos did not have the level of cellular proliferation or apoptosis within cells of the genital ridge as was present in the wild-type embryos, suggesting that Lhx9 may be necessary for the proliferation of gonadal cells, especially cells that are proliferating within the coelomic epithelium during transition of the genital ridge into the indifferent gonad [27]. In mice lacking Lhx9, germ cells migrate normally, but somatic cells of the genital ridge fail to proliferate so a discrete gonad does not form. Also, in Lhx9-deficient mice (as stated previously) there is reduced expression of Sf1 that is essential to the production of both adrenal and gonads within mice [27]. This means that Lhx9 may lie upstream of Sf1 in the developmental cascade of events that occur during testis development.

Emx2 is a mouse homologue of the *Drosophila* head gap gene ems [42, 43]. Emx2 mutant mice die due to failure of the urogenital system to develop.

Emx2 mutants do not have kidneys, ureters, gonads, and genital tracts; however, the adrenal glands and bladder develop normally. Expression of Emx2 was found in the genital ridge next to the Wolffian duct at 9.5 dpc (Fig. 4.2). Wolffian duct development in 10.5-dpc mutant embryos was normal and the mesonephric tubules adjacent to the Wolffian duct were also similar to wild-type. At 11.5 dpc degeneration of the Wolffian duct was observed in several sites and the normal thickening of the coelomic epithelium did not occur in mutants [25]. There was no change in expression of Wt1 in Emx2 mutants, demonstrating that Emx2 expression was not critical for Wt1 expression. However, like Wt1 deficient mutants, the coelomic epithelium of Emx2 mutants did not proliferate and many cells throughout the gonad went through a rapid apoptosis [25]. Therefore, Emx2 may be important in proliferation of the coelomic epithelium, differentiation of cells from the coelomic epithelium, or survival of gonadal cells that are derived from this cell origin.

B. Stage II: Development of Bipotential Gonad

The second stage of gonad formation is development of the bipotential or indifferent gonad (Fig. 4.1). As discussed previously the indifferent or bipotential gonad arises through a thickening or proliferation of cells on the ventromedial surface of the mesonephros directed presumably by genes that are upregulated around 9 to 9.5 dpc. The indifferent gonad becomes visible around 10 dpc in the mouse (other species time points are given in Table 4.1) and can be distinguished from the mesonephros but cannot be identified as a testis or ovary [4]. The indifferent gonad is unique because it has the capacity to differentiate into either the testis or the ovary, depending on transcription factors expressed within the differentiating somatic cells of the coelomic epithelium. Commitment of the indifferent gonad to either the testis or the ovary occurs after migration of primordial germ cells from extragonadal origins to the gonadal ridge. Therefore, the indifferent gonad is composed of primordial germ cells and a thickened layer of coelomic epithelium prior to sex determination.

1. Primordial Germ Cell Migration

Primordial germ cells are the progenitors of male and female gametes. In mammals, PGCs are of extragonadal origin [2, 44]. In the mouse embryo, PGCs are thought to be derived from a small population of epiblast cells (possibly 40–50 cells) set aside in the extraembryonic mesoderm at the mid-primitive-streak stage (7–7.5 dpc) [45]. PGCs migrate from the epiblast through the posterior primitive streak to the allantoic stalk and then to the dorsal mesentery via the hindgut to colonize the urogenital ridges [46–50] in the mouse around 9–11 dpc (Fig. 4.1 and Table 4.1).

The migration of PGCs is composed of four distinct phases. In the first phase, PGCs are passively carried from the yolk sac into the hindgut as a result of embryonic folding. During the second phase, the PGCs leave the hindgut and enter the dorsal mesentery. From the dorsal mesentery, the PGCs migrate by amoeboid movement between the mesenchymal cells of the dorsal mesentery toward the genital ridges to complete the third phase. The fourth phase is the colonization phase, in which the PGCs arrive at and populate the genital ridges [51]. As PGCs migrate they proliferate rapidly and their number increases to around 25,000 at 13.5 dpc [47] in the gonads.

2. Contribution of Extracellular Matrix Proteins to PGC Migration

The migration of the PGCs was initially thought to be primarily through amoeboid movement [52] in many different species [51, 53–62] from the rat and mouse to humans. However, current evidence suggests that extracellular matrix components may be responsible for defining the pathway and pattern of PGC migration. The migrating PGCs can be morphologically characterized by their large, heterochromatic nuclei, the presence of filiform and lobate pseudopodia, microfilaments, and polarized cytoplasm and by expression of alkaline phosphatase [63].

As they migrate PGCs are frequently connected to each other by cytoplasmic threads [64], which may aid in transporting them along the migration pathway. Furthermore, transient interactions between fibronectin molecules on the extracellular matrix and corresponding receptors on the PGCs may also induce germ cell movement [65–67]. Laminin, type IV collagen, chondroitin sulfate [68], and hyaluronan [69] also line the migratory pathway of PGCs and are expressed in the basal laminae surrounding the migration route. Therefore, many extracellular matrix proteins and specifically proteoglycans and glycosaminoglycans may interact to contribute to the migration of PGCs along their route to the genital ridges.

3. Contribution of Growth Factor Secreted Chemoattractants to PGC Migration

A major method of PGC movement has been determined to be through chemoattractants secreted from the urogenital ridge or secreted by cells along the PGC

migratory pathway. Kit Ligand [KL; also called stem cell factor (SCF), mast cell growth factor (MGF), and steel factor (SF)] has been demonstrated to be an important growth factor in PGC migration. Kit Ligand is the product of the murine Steel (SL) gene, and its receptor, c-kit, is the product of the murine White spotting (W) gene. By *in situ* hybridization analysis and histochemical studies of mouse embryos, it has been demonstrated that c-kit transcripts and protein are expressed in migrating PGCs. Also Kit Ligand mRNA expression is associated with cells present in the migratory pathways of PGCs [70–72]. Mouse embryos with mutations at either W or Sl loci show a greater than 99% decrease in the number of PGCs. The deficiency of PGC numbers in Sl/sl embryos was correlated to either a failure in proliferation or an excessive rate of cell death rather than defects in migration. In addition to stem cell factor, transforming growth factor beta 1 has also been demonstrated to induce PGC migration to the genital ridge [73].

4. *Germ Cell–Sertoli Cell Interactions Affecting Germ Cell Mitosis*

Germ cells behave very differently during male and female gonadogenesis. In both sexes, the germ cells migrate to the coelomic epithelium of the gonad between 9.5 and 11 dpc, and they proliferate there until 12.5 dpc (Table 4.1). At this stage in the male, the germ cells are enclosed within the testis cords and enter mitotic arrest. The germ cells in females continue to undergo mitosis until 13.5 dpc when they enter meiotic arrest [74].

The interaction between germ cells and Sertoli cells is thought to play a critical part in sex determination. The Sertoli cell is hypothesized to produce factors that prevent male germ cells from entering meiosis. Initially, it was proposed that enclosure of germ cells within the XY gonads during cord formation inhibited meiosis because PGCs enter meiosis I when they develop in the adrenal gland [75] or when cultured *in vivo* [74]. When seminiferous cord formation was disrupted by cyclopamine in testis organ cultures [76] or in Desert hedgehog (Dhh) null mice XY gonads on a mixed genetic background [77], it was determined that enclosure of the PGCs in cords was not essential for inhibition of germ cell meiosis. Instead it was concluded that inhibition of meiosis was dependent on a diffusible factor originating from the Sertoli cell. One such factor, prostaglandin D_2, has been reported to be produced by Sertoli and germ cells to function in Sertoli cell differentiation and potentially in inhibiting germ cells' entrance into meiosis. This factor, prostaglandin D_2, has also been demonstrated to masculinize female urogenital ridges [78]. Regardless of their chromosomal makeup, germ cells within the gonad will enter meiosis by 13.5 dpc if there is no interference by Sertoli cell secreted factors. Thus, in the female, germ cells will enter meiosis and the gonad will follow normal ovarian differentiation.

The entrance into meiosis by the germ cells may alter the ability of the gonad to form a testis. There is recent evidence that meiotic germ cells may produce factors that inhibit the mesonephric cell migration necessary for formation of seminiferous cords. At 14.5 dpc mesonephric cell migration could not be induced into normal XX gonads, but cells did migrate into germ cell–depleted XX gonads [79]. Even though germ cells are not necessary for induction of testicular cords, they can inhibit migration of cells into the gonad after they have entered meiosis. Therefore, Sertoli cell–germ cell interactions are important during testis morphogenesis, and further elucidation of their interactions is necessary to understand mechanisms regulating abnormal gonadal morphogenesis (e.g., ovotestis formation).

5. *Transcription Factors Expressed in the Indifferent/Bipotential Gonad That Potentially Direct Testis Differentiation*

Several genes are expressed around 10.5 dpc in the indifferent gonad that appear to be important in subsequent sex determination and gonadal morphogenesis. The four most critical genes to testis development that are expressed at this time are Sry, DAX-1, Dmrt1, and Wnt4 (Fig. 4.2). Other genes that are potentially downstream of Sry, DAX-1, Dmrt1, and Wnt4 are discussed later.

Sry was identified as the potential testis determining factor when its translocation from the Y chromosome caused sex reversal [80]. Sry is a member of a transcription factor superfamily that contains a DNA-binding domain call the high-mobility group (HMG) box. HMG box-containing proteins bind and bend DNA acting as architectural transcription factors. The HMG box is the only region within Sry that is conserved among species, and in humans sex-reversing mutations cluster around the HMG box region of Sry [81]. Therefore, it is thought that the HMG region of Sry is critical for testis determination [80, 82, 83]. In rodents, Sry expression is tightly regulated during sex determination [84, 85], whereas in other species Sry may be expressed in many tissues throughout adult life [86, 87]. (Initial Sry expression for different species is presented in Table 4.2.)

Sry expression is potentially regulated by Wt1. Wt1, as discussed previously, is a transcription factor containing four contiguous C_2H_2–type zinc-finger motifs that act as DNA–RNA binding or protein–protein interaction domains. Recent research has determined

TABLE 4.2 Initial Embryonic Testis SRY Expression

Species	SRY/Sry	Cord formation	Reference
Human	7 weeks gestation	7–8 weeks	Hanley *et al.*, 2000; Sinisi *et al.*, 2003
Mouse	10.5 dpc	11.5 dpc	Koopman *et al.*, 1990; Hacker *et al.*, 1995
Rat	12 dpc	13.5 dpc	Berta *et al.*, 1990; Gubbay *et al.*, 1990; Jager *et al.*, 1990; Jost *et al.*, 1981
Pig	E21–20 dpc	26 dpc	Daneau *et al.*, 1984; Parma *et al.*, 1999; Pelliniemi *et al.*, 1981
Sheep	23 dpc	30 dpc	Payen *et al.*, 1996

that Wt1 binds to and acts synergistically with SRY to activate transcription. It is believed that the Sry–Wt1 interaction is mediated by the Wt1 zinc-finger domain and the Sry HMG box [88].

Dmrt1 is expressed around 10.5 dpc in the bipotential gonads of both sexes (Fig. 4.2). After 12.5 dpc transcripts of Dmrt1 are localized to Sertoli and germ cells and its expression is sexually dimorphic at this time, having very weak expression in the ovary after sex determination [89]. Dmrt1 is the best-conserved sex-determining gene between phyla because c-elegans (mab-3) and Drosophila (dsx) have homologous genes that are involved in sex determination [90].

Although the sex-specific expression of Dmrt1 has been established, the role in sex determination has not been determined. Humans carrying deletions in the region of the chromosome containing the Dmrt1 gene are XY sex-reversed [91]; however, mice carrying homozygous deletions of Dmrt1 are not XY sex-reversed but are infertile due to defects in germ cell proliferation and testis development. Dmrt1 does not appear to be important in ovarian development because Dmrt1 mutant females are fertile [92]. The different phenotypes in the human and mouse models may be due to additional gene deletions occurring on chromosome 9 [93].

Currently there are seven different DM genes, termed Dmrt1–7 and several other DM genes; Dmrt3 and Dmrt2 are located adjacent to Dmrt1 on chromosome 9. Deletion of several of these genes in the human model may explain the sex-reversal phenotype, which does not appear to occur within the Dmrt1 mutant mice. Recent expression data have demonstrated that of the seven DM genes three are expressed in the mouse gonad, with Dmrt4 being expressed at similar levels in the testis and ovary. Dmrt3 is more highly expressed in the testis, and Dmrt7 is expressed at higher levels in the ovary [93]. Several Dmrt genes may be involved in gonadal sex differentiation and may be able to partially compensate for other DM gene functions.

DAX-1 (Dosage-sensitive sex-reversal-adrenal hypoplasia congenital-critical region of the X chromosome, gene 1) is expressed around 10.5 dpc in the mouse indifferent gonad (Fig. 4.2). Its expression is upregulated in both sexes from 11.5 dpc and is downregulated in males at 12.5 dpc, but remains in females throughout ovarian development [94, 95]. It was originally proposed that DAX-1 was not necessary for normal testis differentiation. However, recent research with Nr0b1 (DAX-1)-deleted mice crossed to *Mus domesticus poschiavinus* mice (a strain known to be susceptible to XY sex reversal because of an altered Sry allele) demonstrated that DAX-1 is necessary (with normal Sry expression) for testis differentiation [96].

DAX-1 appears to be a dosage-sensitive gene because XY individuals with duplications in DAX-1 show male-to-female sex reversal [97]. In the indifferent gonad, DAX-1 is expressed in the somatic cells, but after sex determination its expression is restricted to the Leydig cells of the testis and thecal cells of the ovary during late embryonic development and adulthood [94, 98]. DAX-1 is proposed to be an antagonist of Sry function in males because both genes are expressed in the same tissues at a similar time period [95]. Coexpression of DAX-1 and Sry in XX mice results in females, whereas XX mice carrying only the Sry transgene develop as males [95]. An early increase in DAX-1 expression is also thought to cause female development in individuals who have Sry expression. The timing of expression of these two genes appears to have dramatic effects on gonadal morphogenesis.

Another function of DAX-1 appears to be in regulation of Sf1. Most *in vitro* studies support a role for DAX-1 in the inhibition of Sf1-mediated gene transcription [99, 100], but the exact nature of their interaction is still unclear. Both Sf1 and DAX-1 are expressed in similar tissues—adrenal gland, gonad, hypothalamus, and pituitary—so it is possible that they may interact to regulate the development of all of these organs [1].

Wnt4 is a member of the Wnt signal transduction family and was originally identified as a mammalian homologue of the *Drosophila* wingless gene. The wingless family regulates paracrine interactions leading to the development of both the kidney and angiogenesis of many different organs [101–104]. Wnt4 is proposed

to antagonize male development in a dosage-dependent manner and to be necessary for ovarian development. Overexpression of Wnt4 leads to upregulation of DAX-1. So Wnt4 in concert with DAX-1 may regulate female gonadal development [105]. Similar to the DAX-1 mutants, Wnt4 knockout mice exhibit similar defects in both kidney and adrenal gland development. Gonadal development and steroidogenic function are affected exclusively in the Wnt4 null females [16]. Wnt4 null females are masculinized and have Wolffian duct structures in the absence of a Müllerian duct system. They also express steroidogenic enzymes required for production of testosterone such as 3β-HSD and 17α-hydroxylase. In normal XX females, Wnt4 may be expressed to suppress androgen biosynthesis so the Wolffian duct structures would not be maintained [16]. In the absence of Wnt4, androgens would be expressed and the Wolffian duct would remain. The absence of the Müllerian duct in the Wnt4 null females is harder to explain. An antagonistic interaction may exist between Wnt4 and MIS in that if Wnt4 is not present MIS can be secreted to initiate regression of the Müllerian duct.

Overexpression of Wnt4 also causes disruptions in gonadal morphogenesis, but in this case it appears to be in the XY gonad. In transgenic mice overexpressing Wnt4, abnormal development of male gonadal vasculature and testosterone production occurred [106], but there was no XY sex reversal. During testis development the formation of the coelomic vessel and organization of blood vessels around the seminiferous cords is one of the earliest morphological sex-specific events. It appears that Wnt4 may affect the ability of blood vessels to develop side branches, resulting in abnormal vascular development [106] in the developing testis. In summary, Wnt4 expression and overexpression appear to antagonize testis development and testis morphogenesis, resulting in either no testis formation or abnormal testis development, respectively.

C. Stage III: Sex Determination

Sex determination in the testis occurs when Sry in conjunction with other transcription factors is expressed within the pre-Sertoli cells to initiate a cascade of events resulting in the formation of a testis. Several elegant experiments using XX mice overexpressing an Sry transgene have demonstrated that Sry is required for normal testis development [107]. Sry appears to be important in at least two critical stages of testis development. The first involves differentiation of the Sertoli cell lineage [108–110] and the second induction of testicular morphogenesis, resulting in formation of testicular cords [107, 111] (Fig. 4.1).

Sry expression within the bipotential gonad regulates expression of genes that differentiate the Sertoli cell lineage from precursor somatic cells in the coelomic epithelium [10.75–11 dpc; 10–15 tail somites (ts)] [112] (Fig. 4.1). The initial differentiation of the Sertoli cell lineage from the coelomic epithelium is a critical step in testis differentiation. The Sertoli cell is the first cell of the testis to differentiate [113], and the Sertoli cells are the only cells in the testis that demonstrate a bias for the presence of a Y chromosome [114]. After differentiation, the Sertoli cells begin to proliferate (11.25 dpc; 16–18 ts), doubling in number at Sry peak expression (11.5 dpc; 18 ts) [107]. Simultaneously, the Sertoli cells move into the gonad proper, forming aggregates with primordial germ cells. Proliferation of the Sertoli cells increases the size of the testis (with respect to the ovary) and appears to be solely dependent on the expression of Sry [107, 115] or Sry-regulated genes. Differentiation and proliferation of the Sertoli cells in the indifferent gonad is required for testis development. (See Table 4.2 for initial Sry expression in other species.)

1. Sertoli Cell Origin

The origin of PGCs has been established as cells that migrate from the base of the allantois via the gut mesentery and colonize in the gonad between 10.5 and 11.5 dpc [45, 64]. Peritubular myoid cells and endothelial cells have been demonstrated to be derived from the migrating mesonephric cells [5]. However, the origin of the Sertoli and Leydig cells has been controversial for some time. Initially, Sertoli cells were thought to be derived from mesonephric cells that migrated into the gonad during testis differentiation and/or from the coelomic epithelium [116]. Differentiated Sertoli cells have been determined to be present within the gonad by 11.5 dpc since MIS, the first known Sertoli cell–secreted hormone, was detected in media from testis cultures at 11.5 dpc [117].

The mesonephros is a mesenchymal tissue derived from the intermediate mesoderm containing the epithelial mesonephric duct and mesonephric tubules. Ultrastructural and staining similarities have been revealed through electron microscopy studies between Sertoli cells and mesonephric tubule cells at the mesonephric–gonadal junction [118–121]. Based on these similarities, it was suggested that the mesonephric tubule cells dedifferentiate from the epithelial structure at the mesonephric–gonadal junction, migrate into the gonad, and contribute to the Sertoli cell population [118–121]. However, no direct experiments have been conducted that support this theory.

2. *Arguments Against the Sertoli Cell Being Derived from the Mesonephros*

Migration experiments using a lacZ transgenic mesonephros (ROSA26) [122] placed in apposition to a wild-type gonad demonstrated that mesonephric cell migration occurred into the male gonad between 11.5 and 16.5 dpc. However, in these experiments Sertoli cells never migrated during this time frame. Instead the cell types that migrated were peritubular myoid, endothelial, or endothelial-associated cells [6]. Therefore, if Sertoli cells do arise from the mesonephros, they must migrate prior to 11.5 dpc. Evidence from Pax2 null mutants would suggest that Sertoli cells do not originate from the mesonephros. In these mutants mesonephric tubules never develop into mature tubules or make contact with the cells of the precursor gonad; however, testis organogenesis proceeds normally with all representative cells present, including Sertoli cells [123].

3. *Evidence That Sertoli Cells Originate from the Coelomic Epithelium*

The coelomic epithelium has also been implicated as a source of Sertoli cells due to its ultrastructural similarities from EM studies [60, 123, 124]. The coelomic epithelium is a single layer of cells that covers the entire coelomic cavity, including the morphological structures that will become the gonad. In the chick, experiments using India ink demonstrated that coelomic epithelial cells migrated into the gonad comprising portions of the developing testis [125]. In the rat, the basement membrane components collagen I and III were fragmented beneath the coelomic epithelium at the onset of gonadogenesis [126]. Both of these observations would suggest that cells from the coelomic epithelium move into the testis and are precursors of cell populations within the testis.

In more recent experiments, the cells of the coelomic epithelium were directly labeled using a fluorescent lipophilic dye, DiI. These cells were imaged and their fate was determined between 15 and 30 ts corresponding to 11.2–12.5 dpc. The coelomic epithelial cells moved to the interior of the gonad and became Sertoli cells as well as other cell lineages. Prior to 18 ts, cells of the coelomic epithelium become pre-Sertoli cells within the seminiferous cords. After 18 ts, labeled coelomic epithelial cells no longer became Sertoli cells but were always found outside testis cords, suggesting that they become a portion of the interstitial cell population. Movement of cells from the coelomic epithelium to the interior portion of the gonad decreased around 12.5 dpc and ended as the tunica albuginea began to form in the XY gonad. Therefore, at least a portion of the Sertoli cell population originates from the coelomic epithelium during early testis differentiation, and later, the cells of the coelomic epithelium become populations of cells within the interstitium [112].

4. *Sex Determination Genes and Sertoli Cells*

The Sertoli cell plays a prominent role in sex differentiation. Through experiments conducted with XX–XY chimeras it was determined that Sertoli cells are the only cell type within the testis required to have a Y chromosome for normal testis development [114]. Sertoli cells express Sry, the male sex-determining gene, and are thought to control male development by influencing the differentiation of other cell types in the XY gonad [95, 127, 128].

Other genes in the sex determination pathway have been studied to determine their function during testis differentiation. Of these genes, *Sox9* (Sry-related HMG box-9) has received the most attention [129]. Sox9 is basally expressed prior to Sry expression [130], but is upregulated just prior to testis morphogenesis [131]. Most evidence would suggest that Sry upregulates Sox9 expression; however, there have been no experiments that demonstrate direct regulation of Sox9 by Sry. Overexpression of Sry or Sox9 causes sex-reversal in XX mice [13, 132]. Individuals with mutations in either Sox9 or Sry will develop as XY females [80, 133]. Sox9 has also been demonstrated to directly regulate MIS in the presence of SF-1 [134]. MIS causes regression of female reproductive tract structures and is one of the first Sertoli cell markers within the testis. Therefore, Sox9 may be as critical to testis development as Sry.

The Sox9 gene may not be without its compensatory molecules. Another Sox gene, *Sox8*, has a similar overlapping expression pattern to Sox9 during mouse testis development [135]. Sox8 also has been determined to regulate the MIS gene, in a manner similar to Sox9, acting synergistically with Sf1. Both Sox8 and Sox9 appear to have arisen from a common ancestral gene and may have retained common functions during testis determination [135]. Sry appears to upregulate several genes such as Sox9 and Sox8, which may compensate for each other during testis development.

The *absence of estrogen* and estrogen's actions may be necessary for testis development. Estrogen receptor double knockout XX mice (without either an α or β receptor) experience female-to-male sex reversals [136]. The gonads of these sex-reversed mice display an overall increased expression of Sox9 [137, 138]. On further characterization of the somatic cell type within these ovaries, it was determined they express Sertoli

cell–specific genes Sox9, TIF1β [139] and TIF2 [140] in both prepubertal and adult ovaries [138]. Therefore, estrogen may be responsible for suppression of testis-specific genes and maintenance of granulosa cell differentiation within the ovary. Further investigation is necessary to determine if the somatic cells within the estrogen receptor double knockout XX gonads are truly Sertoli cells or a cell type that is somewhere in the differentiation pathway between granulosa and Sertoli cells.

The *GATA* family of transcription factors has been shown to be important in the regulation of genes directing differentiation in multiple organs. In the testis and ovary of the mouse, three members of the GATA family are expressed: GATA-1, 4, and 6. Only *GATA-4* is expressed during gonadal differentiation and is present in the somatic cells of the bipotential gonad of the mouse at 11.5 dpc (Fig. 4.2). GATA-4 appears to be expressed in a sexually dimorphic manner with high levels of expression in Sertoli cells of the differentiating testis and reduced expression in the developing ovary [141]. In the pig, expression of GATA-4 is in the coelomic epithelium as it migrates and proliferates to form the indifferent gonad, making this gene a good marker for early gonadal development [21].

The function of GATA-4 during gonadal development remains to be determined. However, GATA-4 was found to activate the MIS promoter through a Gata element that appears to be highly conserved in several species [141]. Expression profiles of both GATA-4 and MIS also appear to be coordinately regulated with expression of GATA-4 preceding that of MIS in both rodents [141] and pigs [142]. Thus, GATA-4, along with several other transcription factors Sf1, Wt1, and DAX-1 [100, 143–145], may be important in regulation of MIS gene expression and Müllerian duct regression within the developing male.

Normal function of the GATA transcription factors requires interaction with zinc-finger proteins of the FOG (Friend of GATA) family [146, 147]. One member, FOG-2, is expressed in the developing mouse gonads by 11.5 dpc [148–150]. FOG-2 null mutants die at 14.5 dpc due to cardiac defects. Partial rescue of cardiac function using a cardiac alpha myosin heavy chain driven FOG-2 (αMHC-FOG-2) transgene expressed in the myocardium extends their life span to 17.5 dpc [151]. GATA-4 null mutants die at 7–9 dpc, whereas the use of a GATA-4 knockin allele (GATA-4^{ki}) allows for interaction with FOG-2 and extends the life span of these mice until 13.5 dpc, at which time altered gonadal morphogenesis can be determined [152].

Testis morphogenesis in FOG-2 null αMHC-FOG-2 transgenic fetuses was abnormal and resembled XX ovaries. Examination of the gonadal histology just after cord formation confirmed that no cords formed in FOG-2 mutant XY gonads. Expression of Sf1 and Wt1 appeared to be normal but expression of Sry was significantly reduced (only 25% of controls). Expression of other Sertoli cell–specific genes, Sox9, MIS, and Dhh, was absent in both FOG-2 and GATA-4^{ki} mutant XY gonads, indicating that Sertoli cell differentiation did not take place. The expression of P450scc, 3β-HSD, and P450c17 were undetectable in both FOG-2 and GATA-4^{ki} mutant XY gonads [153], suggesting that Leydig cell differentiation did not occur either. Therefore, both FOG-2 and GATA-4 are important in Sertoli cell differentiation, seminiferous cord formation, and Leydig cell differentiation. It is possible that both of these transcription factors interact physically with Sry or other genes to initiate enhanced expression of Sry. Without normal expression of Sry, Sertoli cell differentiation and testis morphogenesis cannot occur.

D. Stage IV: Induction of Testicular Morphology and Seminiferous Cord Formation

The formation of testicular cords is a key process in establishing the adult testis morphology. The formation of the seminiferous cords, and potentially the formation of sex-specific vasculature, occurs around 11.5–12 dpc (23–25 ts) and is complete by 12.5 dpc (30 ts) [6, 107, 154, 155] (Fig. 4.1) in the mouse. At this time, the Sertoli cells have ceased proliferating [107] and induction of cord formation has been initiated by migration of cells from the adjacent mesonephros into the developing testis to surround the PGC–Sertoli cell aggregates [5, 117]. Mesonephric cell migration is hypothesized to be the result of Sry-regulated expression of paracrine growth factors secreted by Sertoli cells. Paracrine growth factors secreted by Sertoli cells [6, 154, 155] are speculated to act as chemoattractants to induce mesonephric cells to migrate and surround Sertoli cell–germ cell aggregates within the developing testis [156–161]. As the peritubular and mesonephric cells migrate, they may also secrete factors that enable cells within the testis to morphologically differentiate.

The ovary of the mouse is basically quiescent at this time and does not form follicular structures until several days postnatally in the rodent. Therefore, formation of seminiferous cords is the first morphological indication of testis differentiation and is a result of Sry gene expression. The process of seminiferous cord formation is reliant on factors produced entirely by the testis. The gonadotropin receptors for follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are not present in the rat testis until approximately 16–17 dpc

of testis development [162–164]. Furthermore, the beta subunits for both FSH and LH are not produced by the developing pituitary until approximately 16–17 dpc of embryo development in the mouse [165] with similar later time periods of gonadotropin subunit expression in pigs (after 50 dpc) [166] and sheep (around 70 dpc) [167]. Thus, a paracrine growth factor produced within the testis and triggered by transcriptional regulators such as Sry must be the initiator of testis morphogenesis.

1. *Morphological Changes in Sertoli Cells during Sertoli Cell Differentiation and Aggregation*

An initial step in cord formation is aggregation of Sertoli cells with PGCs. For this aggregation to occur, the Sertoli cells must differentiate and undergo a mesenchymal-to-epithelial cell transformation and polarization [168, 169]. Polarization of the Sertoli cells occurs through accumulation of extracellular matrix proteins such as collagen type IV, laminin [170], fibronectin [171], and heparin sulfate proteoglycan [172] along the basal surface. This correlates with localization of cytokeratin, vimentin, and actin in the basal cytoplasm [170, 173, 174]. As the Sertoli cells differentiate, a number of morphological changes occur including a change in expression of mesenchymal to epithelial cell markers from vimentin to cytokeratin [171] and a change in expression of cytokeratin 19 to cytokeratin 18 (cytokeratin 19 is expressed in the ovary) [175].

In the rat, early gonad expression of laminin α5 and β1 chains are present, but after formation of the testicular cords the laminin α5 chain rapidly disappears. Therefore, the laminin α5 chain can be used as a sex-specific marker for Sertoli cells prior to differentiation into an epithelial cell type. The absence of a laminin α5 chain in the testis can be used to determine that Sertoli cells have undergone differentiation [176, 177]. In addition to laminin, desmin can be used as a marker for Sertoli cell differentiation. In the indifferent gonad, desmin is expressed, whereas after cord formation desmin is no longer expressed within the Sertoli cell [176, 177].

Aggregation of Sertoli cells prior to formation of cords in the testis also requires changes in adhesion of cells to each other and to the extracellular matrix. Both integrin subunits [178] and lectins [179] have been demonstrated to be involved in the early steps of cell aggregation leading to cord formation. Disruption of cell polarization or basal lamina formation by inhibition of protein glycosylation or disruption of actin filaments also prevents cord formation [168, 169]. In addition, treatment of testis organ cultures with retinoic acid [180, 181], or increasing cAMP [182], disrupts formation of the basement membrane and perturbs formation of seminiferous cords.

2. *Genes Regulating Initial Sertoli Cell Transition and Aggregation*

The genes that regulate the process of Sertoli cell mesenchymal-to-epithelial cell transition and Sertoli cell aggregation are yet unknown but are thought to be induced by Sry expression. Lhx9 is downregulated as the Sertoli cells form seminiferous cords and initiate secretion of MIS [27]. Recent experiments have localized an increase in Sox9 expression [183, 184] to Sertoli cells after they have aggregated with the PGCs. Therefore, transcription factors that initiate Sertoli cell transformation may also allow for enhanced expression of Sox9 and reduced expression of Lhx9.

As the Sertoli cell mesenchymal-to-epithelial transition takes place, a decline in laminin α5 chain gene expression and a significant increase in MIS production occur. Later after birth, when MIS expression diminishes and disappears, expression of the laminin α5 chain increases. Thus, it is possible that transcription factors that positively regulate the laminin α5 chain also negatively regulate MIS [177]. Overall, several extracellular matrix proteins can be used as markers to determine if Sertoli cells have made the transition from mesenchymal to epithelial cell types in the developing testis. Of those extracellular matrix proteins, the most promising appear to be the laminin α5 chain and cytokeratin 19.

3. *Mesonephric Cell Migration*

A second step in seminiferous cord formation is the migration of mesonephric cells from the adjacent mesonephros into the differentiating testis (Fig. 4.1). Sry expression is necessary in order to have normal mesonephric cell migration [6, 111]. Removal of the mesonephros prior to mesonephric cell migration results in no seminiferous cord formation. Therefore, the mesonephric cell migration appears to be crucial to testis development. If migration of mesonephric cells is blocked, or if the mesonephros is removed, no seminiferous cords will form [5, 117]. Also, delayed cell migration leads to cord formation occurring at a later time period as seen in B6 XY^{AKR} mice. Alternatively, in B6 XY^{POS} mice, which have reduced Sry expression, cell migration is nearly absent and these mice form ovaries or ovotestes [113].

Mesonephric cell migration is directed by cells within the testis. This was elegantly demonstrated in experiments where mesonephric cells migrated through ovaries toward testis explants [6]. Therefore, these

experiments support the concept that a cell type in the testis induces mesonephric cell migration. The Sertoli cell is the logical candidate to produce paracrine growth factors, which induce mesonephric cell migration [6, 155]. This was concluded since the only other cell type present is the germ cell and germ cell–deficient mice have normal cord formation [185]. Several factors have been proposed to be the Sertoli-derived chemokine, due to alterations in seminiferous cord formation *in vitro* [156–158] and altered testis morphogenesis *in vivo* [159, 161]. These factors are discussed in detail later.

Several different cell types from the mesonephros have been proposed to migrate into the differentiating testis including endothelial cells, preperitubular cells, and cells associated with vasculature [5]. It is difficult to distinguish cell types at this point in gonadal development because some of the cell markers have not been established. However, most literature would agree that both endothelial and preperitubular cell types migrate from the mesonephros and are involved in completion of seminiferous cord formation [5, 186, 187]. Whether these cells migrate coordinately in response to a single cell migration factor or independently due to separate cell migration factors is unknown. It is also unclear as to whether preperitubular cell migration is dependent on endothelial cell migration or vice versa.

In addition to preperitubular and endothelial cells, several laboratories have independently reported migration of cells that appear to be Leydig cell precursors [186, 187]. In both experiments, identification of mesonephric cell types during migration was tracked through the use of cocultures with mesonephroi from either ROSA26 (expressing β-galactosidase) or EGFP-transgenic mice in combination with nontransgenic indifferent testes. Migrating cells with either β-gal or GFP were identified through morphology, [187] positive staining for 3β-HSD, or *in vitro* culture characteristics after isolation [186]. The results of these experiments suggested that a portion of the Leydig population originated from mesonephric cells that migrate into the testis prior to cord formation.

Other laboratories have demonstrated that a portion of Leydig or interstitial cells may be derived from the coelomic epithelium. In experiments where cells of the coelomic epithelia were injected with a DiI fluorescent dye, cells migrating after 12.5 dpc were localized to areas outside of the seminiferous cords, indicating that they were precursors of the interstitial or Leydig cell populations [112]. More research is necessary to determine the percentage of cells that originate from either the coelomic epithelium or from the mesonephros and whether those cells become fetal Leydig cells.

The window of opportunity for proper mesonephric cell migration and induction of testis-specific genes also appears to be critical for normal cord formation and testis development. Induction of migrating cells from the mesonephros can occur as late at 16.5 dpc; however, the best time for proper migration within the male mesonephros is before 12.5 dpc. It is postulated that after 12.5 dpc all critical cells from the male mesonephros have migrated and been donated to the adjacent differentiating testis, suggesting that after 12.5 dpc there are no available cells to migrate into the donor gonad within the organ cocultures [154]. In contrast to this theory, events may also that occur with the developing mesonephros that do not allow for cell migration after a certain development period. Differentiation of the male and female reproductive tracts from the Wolffian and Müllerian ducts may prohibit further cell migration from the mesonephros. Or paracrine factors that induce cell migration may not have viable receptors within the mesonephros after a certain period during gonadal development. The establishment of a critical time for mesonephric cell migration may also explain how a delay in the signal for mesonephric cell migration, as observed in lines of mice that form ovotestis, may result in aberrant gonad formation [154].

4. Growth Factors Involved in Mesonephric Cell Migration and Testis Cord Formation

Many experiments have been conducted to identify Sertoli cell–derived growth factors that induce seminiferous cord formation. Seminiferous cord formation is regulated by factors expressed within the testis since neither gonadotropin receptors nor subunits for gonadotropin hormones are present within the testis or produced within the pituitary gland at this time [10, 162, 163, 165]. Logically the Sertoli cell is the presumed cell type that induces seminiferous cord formation because it expresses Sry and is the first cell type to differentiate in the testis. In addition to this information, germ cell deficient mice still have normal cord formation. Therefore, in contrast [185] to the ovary, which is dependent on primordial germ cell migration for follicle formation [188, 189], the testis does not need germ cells to migrate into the developing gonad for cord formation. These observations support the hypothesis that Sertoli cells secrete a diffusible factor that induces mesonephric cells to migrate toward and enclose the Sertoli cell–PGC aggregates within the developing testis. The mesonephric cells as they migrate may also secrete factors that allow for seminiferous cord formation to be completed. The role of specific growth factors is described later.

5. *Fibroblast Growth Factor 9*

The family of fibroblast growth factors encompasses approximately 22 members, which are widely involved in many developmental processes. Expression of fibroblast growth factor 9 (FGF-9) occurs in at 11.5 to 12.5 dpc in the indifferent and differentiating testis of XY mice and is not present at any time in XX mice or in mesonephros from either XY or XX individuals. In FGF-9 knockout mice, the phenotype ranged from testicular hypoplasia to complete sex reversal. FGF-9 mutants also displayed a reduction in coelomic epithelial proliferation and decreased numbers of Sertoli and interstitial cells [159]. Because a certain proportion of Sertoli cells and interstitial cells comes from the coelomic epithelium, any reduction in cells of the coelomic epithelium would also affect the cell types derived from that affected tissue.

FGF-9 was also demonstrated to induce mesonephric cell migration into XX gonads and cause increased Sox9 expression. Furthermore, organ culture experiments that blocked endogenous FGF-9's actions resulted in impaired testis cord formation. FGF-9 mutant embryos had impaired expression of Sox9, and XX organ cultures treated with FGF-9 formed seminiferous cords with increased expression of Sox9. Therefore, FGF-9 may be involved in induction of mesonephric cells into the testis in order to elicit cord formation. Conversely, FGF-9 may affect the ability of Sertoli cells to aggregate or differentiate in order to upregulate Sox9 at cord formation [159]. In either scenario, FGF-9 is a potential candidate gene that could be downstream of Sry to elicit the actions within the testis that are Sry dependent during testis differentiation.

6. *Platelet Derived Growth Factor and PDGFRa*

Platelet derived growth factors (PDGFs) mediate epithelial-to-mesenchymal interactions (cell proliferation, migration, and differentiation) and are generally expressed in epithelial or endothelial cells, whereas the receptors for PDGF are expressed in mesenchymal cells [190]. The PDGF family is composed of four ligands (A, B, C, D) and two distinct receptors: PDGFRα and PDGFRβ. PDGFRα can bind both A, B, and C homodimers and AB heterodimers, whereas PDGFRβ can bind only the B and D homodimers. PDGF-A is expressed in both XX and XY gonads at 11.5 dpc, and by 12.5 dpc it is strongly expressed in Sertoli cells within the seminiferous cords, whereas expression in the XX gonad is diminished. PDGF-B was expressed in both the XX and XY gonads in endothelial cells by 13.5 dpc [191]. In contrast, PDGF-C was expressed at 11.5 dpc in the coelomic epithelium [192], in the gonadal mesonephric boundary, and at lower levels in cells scattered through the gonad.

Inhibitors to the PDGFR signal transduction pathway in rat testis organ cultures caused abnormal or "swollen" cord formation with increases in cord diameter and reductions in total numbers of cords [193]. Similar results were seen in PDGFRα homozygous mutant mice, demonstrating abnormal cord formation with no distinct division between seminiferous cords and interstitial compartments [191]. Furthermore, there were defects in vascular development, mesonephric cell migration, cellular proliferation, and Leydig cell differentiation [191]. Thus, it appears that PDGF ligands and PDGFRα may play important roles during testis development.

7. *Hepatocyte Growth Factor*

Hepatocyte growth factor (HGF) mediates its effects through a tyrosine kinase receptor c-met [194]. In general, during mouse development HGF mediates epithelial-to-mesenchymal transitions [195, 196] and branching morphogenesis of the lung [197] and also appears to be critical in development of the placenta [198, 200]. HGF is expressed in the developing gonad at 11.5 dpc around the time of seminiferous cord formation. A procedural consideration is that mouse organ cultures generally are cultured with 10% fetal calf serum in order for seminiferous cords to develop *in vitro*. In the absence of serum, cord structures will not form *in vitro*. In contrast, in the rat, serum appears to inhibit cord formation, so organ culture experiments are conducted in serum-free defined media [201]. HGF has been demonstrated to support seminiferous cord formation in mouse organs that were cultured without serum [158]. Therefore, the presence of HGF may be necessary or permissive in order for cord formation to occur *in vitro*. In addition, HGF does induce mesonephric cell migration and could be a potential growth factor involved in the process of cord formation in the mouse [202].

8. *Neurotrophin Growth Factor Family*

A role for neurotrophins and their receptors has been implicated in many systems at sites of mesenchymal-to-epithelial cell interactions [203–209]. The four neurotrophin ligands are neurotrophin-3 (NT3), nerve growth factor (NGF), brain derived growth factor (BDNF), and neurotrophin-4/5 (NT4/5) [210]. All neurotrophin ligands bind to a low-affinity receptor p75 neurotropin receptor (p75NTR) and each has more specific high-affinity trk receptors. NT3 binds to trkC,

NGF binds to trkA, and both BDNF and NT4/5 bind to trkB [211–214].

In rat testis, the low-affinity receptor for neurotropins, p75NTR, had been determined to be present in a sex-specific manner in the testis at the time of seminiferous cord formation. Expression of p75NTR was present in the mesonephros prior to cord formation and then was localized to cells surrounding seminiferous cords at cord formation. Later at postnatal day 0 (P0), p75NTR was expressed in the single layer of peritubular cells that surrounding the seminiferous cords [156]. Similar expression profiles were determined in mice [215] and humans [216] for p75NTR during testis differentiation. p75NTR was determined to be a marker for early migrating mesonephros cells that develop into preperitubular cells [217]. NT3 and its high-affinity receptor trkC were expressed in the Sertoli cell and within specific cells of the mesonephros prior to cord formation.

Subsequent experiments determined that anti-sense NT3 oligonucleotides [157] and trkC-IgG fusion proteins had partial inhibition of seminiferous cord formation [156] while both trk signal transduction inhibitors (K252A and tyrophostin AG879) inhibited seminiferous cord formation [156, 161]. The trk specific signaling inhibitor AG879 was shown to inhibit seminiferous cord formation by inhibition of mesonephric cell migration. Furthermore, NT3-coated beads induced mesonephric cells to migrate into XX gonads similar to FGF-9 [161]. Taken together, these results demonstrate that NT3 may be a Sertoli cell–derived chemotactic factor involved in mesonephric cell migration.

Testicular cord formation in both trkC and trkA knockout mice appears to be developmentally delayed with reductions in the number of cords at E14 [160]. In addition, trkC knockout mice had reduced interstitial area at E13 and a reduction in the number of seminiferous cords at E19. Additional germ cell abnormalities were detected with both receptor mutants demonstrating reduced germ cell numbers during embryonic development. Furthermore, P19 trkA knockout mice had increased germ cell apoptosis when compared to wild-type controls. The trkA knockout mice also appeared to have increased aberrant branching of seminiferous cords at E14 and P19.

Double knockout mice (trkC/trkA) did not have increased testis morphological abnormalities when compared to phenotypes of either knockout alone; however, the numbers of double knockouts were low, 2 in 80 embryos, one of which was female [160]. Therefore, absence of all trk receptors may be necessary to determine compensatory functions of the neurotropin ligands and receptors. A similar observation was found in triple insulin receptor knockout mice [218]. In human embryonic testis cultures, treatment with the trk receptor inhibitor K252A resulted in reduced numbers of peritubular cells, Sertoli cells, and gonocytes [216]. Observations support a role for neurotropins in testis cell growth and survival. Therefore, the neurotrophin family appears to have functions in both testis morphogenesis and germ cell development. Sertoli cell–expressed NT3 appears to be a chemotactic factor for the migrating mesonephros cells that are required for testis cord formation.

9. Estrogen or Estrogen receptor

Estrogen receptor double knockout mice (those lacking both the alpha and beta receptor genes) have been demonstrated to display female-to-male sex reversal in XX mice. Furthermore, as discussed earlier, estrogen receptor double knockout mice have somatic cells that express Sox9, which is a Sertoli cell–specific gene. Researchers concluded from these experiments that estrogen was important in suppression of Sox9 expression in somatic cells and without its action this somatic cell linage was capable of expressing Sox9 and initiating the testis development pathway [137].

Both estrogen and androgen receptors are present around the time of seminiferous cord formation in mice [219] and rats [220, 221]. Estrogen may also have adverse effects if expression occurs abnormally in males. Experiments utilizing rat testis organ cultures demonstrated that cord formation was inhibited in a dose-responsive manner when exposed to increasing levels of estrogen. Furthermore, cord formation was also inhibited when organs were exposed to compounds that were antiandrogenic [220]. The effects of estrogen may be directly on Sertoli cell function because estrogen receptor alpha is present in Sertoli cells at this developmental period. However, the antiandrogenic effects may be more indirect because the androgen receptor is localized to germ and interstitial cells around cord formation [220]. Steroid hormones and their receptors are potentially involved in critical morphological gonadal development and maintenance of cell differentiation within the testis and ovary.

10. Insulin Receptor Family

The insulin receptor family composed of insulin receptor (Ir), insulin-like growth factor receptor 1 (Igfr1), and insulin-receptor–related receptor (Irr) is important in multiple cellular pathways to direct growth and metabolism. Single receptor null mutants for Ir die after birth due to ketoacidosis [222, 223] and Ir die at birth due to respiratory failure [224]; whereas Irr mutants are viable and show no phenotype [225]. Testis morphogenesis

and cord formation in single null mutant mice appear to be normal; however, gross morphologic sex reversal (testis to ovary) occurs in triple mutants Ir/Irr/Igfr1. Expression of testis-specific genes, Sox9 and MIS, are absent in mutant gonads with coordinate high expression of ovarian-specific genes, Wnt4 and Figα.

In double null mutants Ir/Igfr1 and compound heterozygous $Ir^{+/-}/Irr^{-/-}/Igfr1^{-/-}$, $Ir^{-/-}/Irr^{-/-}/Igfr1^{+/-}$ knockout mice the degree of sex reversal was more variable with lowered expression of testis-specific Sox9, MIS, and Insl3 and ovarian-specific genes, Wnt4 and Figα. Gonadal cells from the triple knockouts also proliferated more slowly, resulting in a smaller gonad than their wild-type littermates [218]. Reduced proliferation is also critical because proliferation of the coelomic epithelium is dependent on Sry and is necessary for normal development of the XY gonad. Therefore, this research indicates a critical role for the insulin receptor family in testis development and adds yet another growth factor family to the list that is involved in the complex pathway of gonadogenesis.

11. Similar Signal Transduction Pathways That Affect Seminiferous Cord Formation

Several of the growth factors (FGF-9, HGF, NT3, insulin) that are involved in seminiferous cord formation have a common signal transduction pathway that is activated, which is PI3 kinase [226]. All of these growth factors also appear to induce mesonephric cell migration or have a potential role in induction of mesonephric cells into the differentiating testis. Experiments using a PI3 kinase inhibitor specific to the 110 isoforms of class I PI3 kinases demonstrated in rat testis organ cultures (in a dose responsive manner) an inhibition of seminiferous cord formation. These observations demonstrated that a PI3 kinase inhibitor and not a Map kinase inhibitor were critical to seminiferous cord formation through inhibition of mesonephric cell migration. Therefore, the PI3 kinase signal transduction pathway may be a common pathway that multiple growth factors induce through direct or indirect Sry actions to promote mesonephric cell migration and seminiferous cord formation [226].

An inhibitor of the protein kinase A signal transduction pathway, forskolin, also inhibited mesonephric cell migration in mouse gonad cultures. However, there were no effects on Sertoli cell differentiation because Sertoli cells were capable of expressing MIS, Sox9, and Dhh. Forskolin was utilized because the protein kinase A pathway is a signal transduction pathway activated by the Dhh gene within the testis [76]. The Dhh gene is expressed in Sertoli cells during testis development, and its receptor patched1 (Ptc1) is expressed in interstitial, peritubular, Leydig, and endothelial cells [77, 227]. The phenotype of Dhh null mice is variable depending on the genetic background. Some backgrounds have normal testis structure but defective spermatogenesis [227]. In contrast, Dhh mutants from other genetic backgrounds exhibit defects in development of peritubular cells, an absence of basal lamina, apolar Sertoli cells, anastomotic testicular cords [228], and defects in Leydig cell differentiation [77]. Therefore, Dhh may initiate critical steps during testis differentiation.

In summary, both protein kinase A and PI3 kinase appear to be important pathways for regulation of seminiferous cord formation potentially through regulation of cell migration. Previous research has demonstrated that calphostin C, a potent protein kinase C inhibitor, did not have any effect on seminiferous cord formation [156]. Because testis morphogenesis is crucial for male adult testis function, multiple signal transduction pathways may be necessary to ensure proper regulation of seminiferous cord formation within the male gonad.

12. Pattern of Sertoli Cell–Specific Gene Expression and Cord Formation

Sry expression is first detected in the central region of the gonad around 11 dpc and its expression spreads to the anterior and posterior poles within the testis [15, 113]. This suggests that pre-Sertoli cells within the central region of the testis are more advanced than other pre-Sertoli cells within the indifferent gonad at this time. There do not appear to be morphological changes in cells between the regions within the indifferent gonad at this stage. Therefore, there must be differential gene regulation or altered access to factors (potentially through closer vascular supply) that initiate Sertoli cell differentiation and Sry expression.

Like Sry expression, seminiferous cord formation also occurs from the central region of the gonad into the anterior and posterior poles of the gonad. The chronology of testis cord formation also explains the morphology of ovotestes, which comprise ovarian tissue on either side of testicular tissue [15, 113]. Recent experiments have demonstrated different abilities of certain regions of the gonad to form seminiferous cords during development. The central region of the gonad (separated from both the anterior and posterior regions) can initiate cord formation at a much earlier stage in gonadal development (12–14 ts) when compared to both the posterior and anterior regions (15–17 ts) [229] (Fig. 4.3). Therefore, this supports previous conclusions that pre-Sertoli cells in the central portion of the gonad are at a more advanced stage of differentiation, have greater

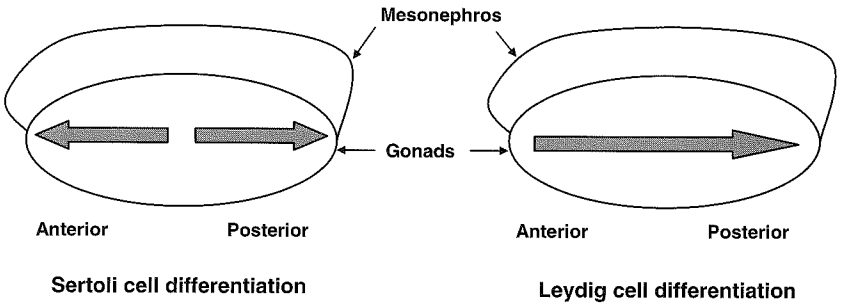

FIGURE 4.3 Regional differences in somatic cell differentiation within the gonad. Arrows demonstrate how each somatic cell type differentiates: Sertoli cells differentiate from center to pole, whereas Leydig cells differentiate from anterior to posterior within the differentiating XY gonad [15, 229].

access to factors that allow them to develop differentiated functions earlier, or have a greater innate capacity to initiate events earlier during testis development.

13. Müllerian Inhibiting Substance/Anti-Müllerian Hormone

MIS is a member of the transforming growth factor β (TGFβ) family of growth factors and is expressed specifically in the Sertoli cells of the testis around 11.5 to 12.5 [230] (Fig. 4.2). The MIS gene is the first Sertoli cell-secreted protein that is produced at or after the time of seminiferous cord formation. The role of MIS in the testis appears to be severalfold. MIS received its name initially because it was the factor that caused Müllerian duct regression and eliminated any precursors of the female reproductive tract. Around 11.5 dpc the mesonephros contains two duct structures that are precursors of the female, the Müllerian duct or paramesonephric duct, and the male, Wolffian duct or mesonephric duct, reproductive tract structures [231, 232]. In the female XX gonad, where no Sertoli cell differentiation occurs, the Müllerian duct will develop and eventually form portions of the female reproductive tract structures. In contrast, in the male, MIS expression by the Sertoli cell will cause regression of the Müllerian duct through initiation of Müllerian duct cell apoptosis. In MIS null male mice, the Müllerian duct fails to regress and both male and female reproductive tract develop [19, 233]. Recently, MIS has been identified as a factor that induces mesonephric cell migration. However, because no abnormalities were found in early testis development within MIS null mice, it is believed that this function of MIS may be redundant and shared with another member of the TGFβ family [234].

14. Endothelial Cell Migration and Development of Vasculature

Sex-specific vascularization and development of a large blood vessel supply occurs in the developing testis (XY gonad) under the coelomic epithelium by 12.5 dpc in the mouse. There is no research available to suggest if testis vasculature develops at the time of seminiferous cord formation or after testis morphogenesis. However, because both endothelial cells and preperitubular cells migrate into the differentiating testis as testis morphogenesis is initiated, it is hypothesized that both seminiferous cord formation and vasculogenesis in the testis occur simultaneously. Therefore, in addition to surrounding the Sertoli cell–PGC aggregates, the migrating mesonephric cells may also initiate the formation of vasculature within the developing testis directed by Sertoli-secreted factors downstream of Sry expression [235].

At 11.5 dpc the endothelial cells express both venous (EphB4) and arterial (Ephrin B2) markers in both the XX and XY gonad, which suggests that vascular development at this time is not sex specific. However, just after 11.5 dpc, vasculature within the developing testis undergoes rapid reorganization, causing the formation of the coelomic blood vessel, organization of endothelial cells to the interstitium outside of the seminiferous cords, and extensive branching of vessels [235]. By 12.5 dpc the vasculature of the testis labeled positive for the arterial-specific marker Ephrin B2, whereas the ovary at this time still has both arterial and venous makers. Sertoli [235] cells have been determined to express many growth factors that cause endothelial cell migration such as vascular endothelial growth factor (VEGF) [236] and PDGF [191, 193]. Therefore, the development of the cell-specific vasculature around the time of cord formation is presumably mediated by Sertoli cell–secreted proteins, which are expressed downstream of Sry expression and Sertoli cell differentiation.

E. Stage V: Development of a Functional Testis

After seminiferous cord formation is completed, the other cells within the testis initiate differentiation. It is proposed that the Sertoli cell continues to differentiate

and proliferate after seminiferous cord formation and that interstitial and Leydig cells differentiate after initial Sertoli cell differentiation. In part, this may be due to factors produced by the Sertoli cells that direct interstitial, peritubular, and Leydig cell differentiation. The peritubular layer of cells becomes identifiable from the interstitial or Leydig cells at 15 dpc in the rat [237]. The peritubular cells become a single layer of cells surrounding the seminiferous cord in rodents and a multilayered cell layer in some domesticated livestock [238]. A major role of the peritubular cell is to form the outer layer of the seminiferous cord and to jointly form with the Sertoli cell the blood–testis barrier to protect haplotype germ cells from any attacking immune cells.

1. *Leydig Cell Differentiation*

In some species timing of seminiferous cord formation greatly impacts Leydig cell differentiation and initiation of steroidogenesis. 3β-HSD production, which is a requisite for androgen production, was detected after 15 dpc in the rat [239] and at 13 dpc in the mouse [240]. In the pig, Leydig cell differentiation was determined to occur at 28 dpc (due to positive staining of cells in the interstitium for P450c17), which was several days after Sertoli cell differentiation and cord formation (26 dpc) [142]. Recently, it was determined that although Sertoli cell differentiation occurs from center to pole (Fig. 4.3), the 3β-HSD–positive cells differentiate from the anterior region of the gonad to posterior. This supports earlier studies where Sf1-positive cells were found in the anterior region of the indifferent gonad and were thought to be precursors of both the adrenal steroidogenic cells and the Leydig cells of the differentiating testis [241].

Furthermore, cells in the mouse gonad stained positive for 3β-HSD at 11 ts, which is much earlier than has been reported. The presence of 3β-HSD indicates that the somatic cells of the testis destined to become Leydig cells are capable of steroidogenesis (specifically testosterone production) at a much earlier time point than previously thought. Testosterone is critical to the developing male because androgens stabilize the Wolffian duct derivatives for normal male duct development [242, 243]. Therefore, appropriate differentiation of somatic cell types in the testis prior to and around the time of cord formation is crucial not only to the normal development of the testis, but also for the continued presence of the Wolffian duct and future development of the male reproductive tract.

2. *Sertoli Cell Proliferation*

Growth and proliferation of cells within the testis are critical for testis morphogenesis. After the process of cord formation, the testis undergoes a dramatic sex-specific growth. At 15 dpc in the rat, the testis is twice the size of the same age ovary [244]. Proliferation of the Sertoli cell population is important because each Sertoli cell can support a finite number of germ cells in the adult testis [9, 245]. An increased number of Sertoli cells correlates with increased spermatogenic capacity. After puberty, the Sertoli cells are no longer mitotic and the population of Sertoli cells is established. The rodent has two critical developmental periods when Sertoli cells are proliferating—during late embryonic development and again during early postnatal testis development [9, 246].

The greatest rate of embryonic Sertoli cell proliferation occurs from 18 dpc to P0 in the rat [247] with proliferation in late gestation as well in the pig [248]. Other testicular cell populations also proliferate from cord formation to birth (from 14 dpc to P0) [247]. At 14 dpc the germ cells, interstitial cells, and peritubular cells are all proliferating rapidly. The Sertoli and peritubular cells continue to proliferate at a high rate at P0, whereas the germ cells experience a dramatic reduction in proliferation. After birth all the cells proliferate until the onset of puberty, when Sertoli cells become postmitotic [247] (Fig. 4.4).

Many factors have been shown to affect cell growth in the embryonic testis and some of the growth factors also affect organization of seminiferous cords. Several growth

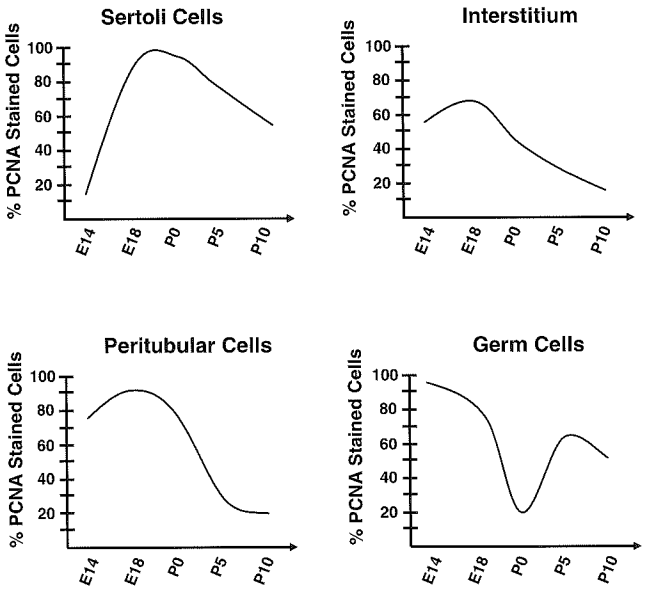

FIGURE 4.4 Proliferation of each cell type within the developing rat testis from E14 through postnatal day 10. Most cell types accomplish most of their proliferation during embryonic development. Proliferation was assessed by percentage of cells staining positively for PCNA [247].

factors that regulate cell growth in the embryonic testis are discussed in the following sections.

3. *Transforming Growth Factor Alpha Family*

The transforming growth factor α (TGFα) and epidermal growth factor (EGF) have been associated with multiple roles during testis development. EGF increases production of inhibin, androgen-binding protein, and transferrin by Sertoli cells [249, 250]. EGF also affects Leydig cell function by altering cholesterol transport and the number of LH receptors and by regulating testosterone production [251, 252]. TGFα has been demonstrated to stimulate peritubular cell proliferation and migration in culture [253, 254]. TGFα mRNA has been detected in cultured Sertoli and peritubular cells [253, 254] and TGFα protein was found in Leydig cells [255]. EGFR, the receptor for both EGF and TGFα was found at the protein level in adult Sertoli and Leydig cells, and at the mRNA level in prepubertal peritubular cells [253, 256, 257].

TGFα and EGF have been demonstrated to increase cell proliferation at the time of cord formation and during late embryonic testis development [247, 258]. Most of these experiments were *in vitro* utilizing TGFα and EGF antagonists and receptor signaling antagonists. Most of the proliferative effects of TGFα appeared to be on Sertoli cells and cells in the interstitium. Knockout mice for EGFR (the receptor for TGFα) demonstrated a reduction in interstitial cells surrounding the seminiferous cords at 18 dpc. These differences were compensated for by birth in these mice [247]. In addition to its effect in the testis, EGF is also important in mediating the effects of testosterone to stabilize the Wolffian duct and allow it to differentiate [242, 243]. Therefore, both TGFα and EGF appear to be important in proliferative and morphogenic processes within the developing testis and reproductive tract.

4. *Transforming Growth Factor Beta Family*

TGFβ isoforms are growth factors that regulate growth and development in many different cell types. All three mammalian isoforms have been identified in the adult testis [181, 259–264]. $TGF\beta_1$, $TGF\beta_2$, and $TGF\beta_3$ have also been localized to the embryonic testis. $TGF\beta_1$ appears in the testis at 14.5 dpc in the rat (after cord formation) and has been localized to the seminiferous cords and interstitium. $TGF\beta_2$ was localized to the Sertoli cells at 14.5 dpc, with localization to the Leydig cells (along with $TGF\beta_1$) at 16.5 dpc [264, 265]. In contrast, $TGF\beta_3$ was localized to preperitubular cells at 14 dpc [181] and to germ cells from 14.5 dpc to birth [181, 266]. This localization of $TGF\beta_3$ suggests that it may be involved in critical processes involved in the restructuring of the gonad and germ cell maturation/proliferation during embryonic testis development. One role of $TGF\beta_1$, $TGF\beta_2$, and $TGF\beta_3$ from 16.5 to 20 dpc is to regulate LH stimulated steroidogenesis from the Leydig cells [266, 267]. $TGF\beta_3$ may be involved in seminiferous cord formation; however, it does not appear that $TGF\beta_1$ or $TGF\beta_2$ is involved in differentiation of the testis, only in testis proliferation.

Gene knockout and overexpression experiments with TGFβ have demonstrated that precise regulation of each isoform is essential for survival. $TGF\beta_1$ null mice are phenotypically normal until approximately 3 wk after birth and then they develop a severe wasting syndrome [268]. Reproductive traits and organs within $TGF\beta_1$ knockout mice have not been extensively studied. However, there are significant deviations from normal mendelian ratios, resulting in decreased offspring for both heterozygotes and homozygotes carrying the allele with the $TGF\beta_1$ gene disruption. $TGF\beta_2$ knockout mice possess abnormalities in urogenital development. These alterations within the reproductive tract ranged from testicular ectopia to unilateral testicular hypoplasia to complete absence of the epididymis [269]. However, these investigators did not conduct an extensive examination of testis histology to determine what processes in testis development were affected with the TGFβ deletion.

TGFβ signals through the interactions of TGFβ receptor I and TGFβ receptor II. TGFβs bind to TGFBRII, which phosphorylates TGFβRI, causing the [270] activation of the SMad family of transcription factors [271]. TGFβRI and II are present in germ cells of mice around the time of cord formation (11.5 dpc) [272]. In the rat TGFβII has also been localized to germ cells at the time of cord formation (13.5 dpc) and later in the Leydig cells (16.5 dpc) where TGFβs may regulate steroidogenesis [267]. A requirement for TGFβRIII has been demonstrated in endocardial cell transformation in the heart where a mesenchymal-to-epithelial transition takes place [273]. Therefore, all three receptor isoforms may be important in mediating the actions of the TGFβ ligand isoforms.

5. *Neurotrophin Growth Factor Family*

As stated previously neurotropin growth factors, specifically nerve growth factor and neurotropin-3, are two neurotropins that are secreted by Sertoli cells early during testis development and prior to cord formation [157, 215]. After cord formation they are localized to other cells and may be involved in germ cell maturation or Leydig cell differentiation. In both trkC and trkA (receptors for NT3 and NGF, respectively) knockout

mice, reductions were observed in the area of the interstitium during embryonic development, which may suggest that the neurotropins are necessary for interstitial cell proliferation. In addition, reductions in germ cells during embryonic development were observed that indicate germ cell maturation/proliferation or survival may be dependent on neurotropins [160]. Both NGF and NT3 demonstrated a dose-dependent increase in incorporation of tritiated thymidine in day 0 testis cultures [157]. Therefore, NGF and NT3 are potential regulators of testis growth during the late embryonic period after cord formation.

6. PDGF

PDGF appears to be important for formation of normal cords as previously discussed. PDGF also appears to affect the differentiation of the Leydig cell population [191]. PDGF was not determined to stimulate growth of P0 testis when experiments were conducted using tritiated thymidine [193]. Therefore, PDGF appears to affect early morphogenesis and cell proliferation events during embryonic testis development.

7. Steroid Hormones and Their Receptors

Estrogens and androgens both have been demonstrated to affect cell growth within the embryonic testis [220]. There also is accumulating evidence that high levels of estrogens and antiandrogens will affect cell growth within the testis and have the potential to cause adult testis abnormalities [274, 275]. Estrogens and androgens, as discussed previously, can have dramatic effects on gonadal development, growth of specific cell populations, and differentiation of the reproductive tracts. Therefore, any abnormal production of these steroid hormones can influence embryonic testis development and adult testis function.

8. Thyroid Hormone

A wealth of information has been collected on the effects of transient hypothyroidism on early postnatal Sertoli cell differentiation. Briefly, transient hypothyroidism postnatally increases Sertoli cell numbers, increases testis size, and increases daily sperm production in mice and rats [276–281] with similar effects found in rams [282, 283] and cattle [10]. Other experiments conducted in the rat to induce hypothyroidism in pregnant females during late gestation demonstrated no effects on fetal testis growth, but resulted in reduced serum levels of gonadotropins and delayed maturation of Sertoli cells and puberty [284]. Recently, two lines of pigs that have divergent adult testis size were evaluated to determine if alterations in endocrine hormones may elicit the differences in testis size.

The Meishan Chinese breed of pigs have a shorter Sertoli cell proliferation period, which results in smaller seminiferous cord diameter, larger interstitial area, and earlier age at puberty when compared to European White Cross pigs [248, 285]. Meishan pigs were demonstrated to be hyperthyroid during the late fetal period, which correlated with the time when Sertoli cell proliferation was reduced. In addition to increases in thyroid hormone, expression of thyroid receptor β_1 was also enhanced at this time, which suggested enhanced testis sensitivity to thyroid hormone. Therefore, high levels of thyroid hormone may shorten Sertoli cell proliferation during late fetal testis development and induce early Sertoli cell differentiation. These alterations in Sertoli cell differentiation [286] may also affect Leydig cell differentiation, resulting in greater interstitial area and reduced area of seminiferous tubules. Further studies are necessary to elucidate the exact mechanisms that regulate thyroid hormone actions on embryonic testis development and morphogenesis, specifically with regard to Sertoli cell proliferation and differentiation.

9. Gonadotropins

FSH and LH receptors are present in the testis during late gestation in many species [10, 162, 163]. FSH and LH have been demonstrated to be critical for postpubertal development and proliferation of Sertoli and Leydig cell populations in the testis [287, 288]. Early experiments demonstrated partial gonadotropin dependence of Sertoli cells during fetal development [246, 289]. Recent experiments suggest that gonadotropins do not play a critical role in regulation of Sertoli and Leydig cell populations in the fetal period [290, 291]. Mice that lack the thyroid-specific enhancer-binding protein (T/ebp or Nkx2.1) gene do not develop the thyroid gland, lung, ventral forebrain, and pituitary gland. Because they do not develop a functional pituitary with normal synthesis and secretion of gonadotropin hormones, they are an excellent model to determine how gonadotropins affect morphological testis development and growth. Male mice lacking the T/ebp/Nkx2.1 gene have a normal male phenotype with normal seminiferous cord formation. The testes from these mice produce lower levels of testosterone, have smaller Leydig cells (after 18.5 dpc), and have slightly smaller testes [291].

These data support earlier reports that seminiferous cord formation is independent of gonadotropins.

Hypogonadal mice demonstrate normal Sertoli cell and Leydig cell differentiation and proliferation during the fetal period [290]. Therefore, gonadotropins do not appear to be critical regulators of embryonic testis proliferation during the late fetal period. Most of the regulation of cell proliferation during fetal development must be through paracrine growth factors produced within the differentiating testis.

IV. SUMMARY

The Sertoli cell has been described as the "organizer" of the embryonic testis. Probably a more descriptive title is "orchestrater" because the Sertoli cell initiates, as well as organizes, testis development. Normal testis development is dependent on Sertoli cell differentiation and expression of genes that direct morphological development along the XY pathway. The Sertoli cell is the somatic cell type that initiates differentiation by expressing Sry, which has been described as the master gene in male development. Certainly Sry is critical for testis development. Several other genes, however, such as Sox9, Dmrt1, and DAX-1, appear to need to be expressed appropriately in order for testis differentiation to occur in the indifferent gonad.

In the past 12 years, since the identification of Sry multiple genes, morphological events have been elucidated to obtain a better understanding of how testis development progresses from the indifferent gonad. However, further research is necessary to understand (1) what genes are immediately downstream of Sry, (2) what additional paracrine growth factors regulate Sertoli cell differentiation and cord formation, and (3) how embryonic Sertoli cell differentiation impacts other events such as Leydig cell differentiation and adult testis spermatogenic function. Future research using technological advancements such as RNAi, laser capture microscopy, tissue-specific gene expression models, and mouse mutagenesis procedures should add to our understanding of factors regulating embryonic Sertoli cell differentiation and testis development.

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"The question of how a testis or an ovary develops from its early embryonic primordium is given a rather simple answer in most elementary textbooks. The question is not as clear if one looks at original papers. For more than a century many very good biologists have discussed the question and debated theoretical interpretations without reaching a general agreement." (Alfred Jost, 1973)

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