

Cellular and Molecular Pathways Regulating Mammalian Sex Determination

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ABSTRACT

In mammals, sex is determined by the presence or absence of a single gene on the Y chromosome, *Sry*. *Sry*, a member of the high mobility group family of transcription factors, is required to initiate male-specific pathways and repress female-specific pathways. Expression of *Sry* in the gonad, beginning at 10.5 days postcoitum, leads to the differentiation of the somatic supporting cell precursors as Sertoli cells. These cells direct the other cells of the gonad into their respective lineages. Currently, no direct targets of *Sry* are known. A number of cellular pathways initiated by *Sry* are required for testis development. These include the proliferation of pre-Sertoli cells and commitment to the Sertoli lineage, migration of cells from the adjacent mesonephros, and formation of a male-specific vasculature. Work is underway to identify genes controlling these processes. These genes will then be linked to *Sry*.

I. Introduction

Organisms that display sexually dimorphic phenotypes have evolved a variety of mechanisms for determining sex. In worms and flies, sex is determined by the ratio of X chromosomes to autosomes (Parkhurst and Meneely, 1994; Cline and Meyer, 1996). Reptiles and fish use hormones, temperature, or environmental cues to determine sex (Shapiro, 1990; Pieau *et al.*, 2001). In mammals, sex is determined genetically by the presence or absence of a Y chromosome (Swain and Lovell-Badge, 1999; Capel, 2000).

In 1947, Alfred Jost showed that removal of the gonads from fetal rabbits resulted in the development of female secondary sex characteristics, whether the animal was XX or XY. These experiments showed that male secondary sex characteristics depend upon the presence of a testis and led to the hypothesis that female development was the “default” state, since no gonads were required to specify female characteristics. Subsequently, the Y chromosome was found to be consistently associated with male sexual development, regardless of the number of X chromosomes present. The hypothetical factor on the Y chromosome required for male differentiation was termed *Tdy* (for testis-determining factor on the Y chromosome).

In 1990, studies performed in humans on sex-reversed XX males revealed that a 35-kb region of the Y chromosome was capable of causing sex reversal when transferred to an X chromosome (Gubbay *et al.*, 1990; Sinclair *et al.*, 1990). A single gene common to all mammals was found in this region and named *Sry* (for sex-determining region of the Y chromosome). Expression studies in mice revealed that *Sry* is expressed in the gonad from 10.5 days postcoitum (dpc) to approximately 12.0 dpc, coincident with formation of the gonad and initiation of testis development (Koopman *et al.*, 1990; Hacker *et al.*, 1995; Bullejos and Koopman, 2001). Further studies showed that when a 14-kb region containing this gene was introduced into XX mice as a transgene, testis development and male secondary sexual differentiation occurred, proving that *Sry* is *Tdy* (Koopman *et al.*, 1991). However, these mice are sterile because other genes on the Y chromosome are required for spermatogenesis.

The *Sry* gene encodes a member of the high mobility group (HMG) family of transcription factors that has been shown to activate transcription by binding and bending DNA (Kamachi *et al.*, 2000). More recent experiments have begun to reveal a role for HMG proteins in transcriptional repression as well (Zorn *et al.*, 1999). Thus, *Sry* may initiate the male pathway by activating testis-specific genes and/or repressing genes that are specifically involved in ovarian development. Other members of this family include *Tcf1* (T-cell factor 1), *Tcf4*, *Lef1* (lymphoid enhancer binding factor), and *Sox* (*Sry*-related box) genes. These genes are involved in a number of developmental processes, ranging from cartilage formation to specification of neural identity (Pevny and Lovell-Badge, 1997).

II. Origin and Development of the Gonads

In the mouse, the urogenital ridge develops from intermediate mesoderm, beginning around 9.5 dpc. This starts with formation of the pronephros at the anterior end of the body cavity. The mesonephros develops centrally and gives rise to the gonads and adrenal glands. The metanephros, the area in which the ureteric bud branches to form the kidney, develops more posteriorly. At 10.0 dpc, the gonads begin to develop on the ventromedial surface of the mesonephroi. The somatic cells of the gonad originate from both the mesonephros and the coelomic epithelium, a single layer of epithelial cells that lines the coelomic cavity (Byskov, 1986; Karl and Capel, 1998). In both XX and XY animals, primordial germ cells (PGCs) first form at 7.5 dpc at the base of the allantois. As the embryo undergoes morphogenesis, PGCs migrate through the gut mesentery and populate the urogenital ridge at 9.5 dpc. The PGCs then populate the gonads as they are beginning to form at 10.0 dpc (Ginsburg *et al.*, 1990; Gomperts *et al.*, 1994). Until 11.5 dpc, the gonads in XX and XY individuals appear identical and are capable of forming either testes or ovaries. By 12.5 dpc, the formation of testis

cords can be seen in XY gonads, whereas no obvious changes occur in XX gonads. XY gonads are noticeably larger than XX gonads by this time and acquire a characteristic pattern of vasculature.

III. Cell Types of XX and XY Gonads

Available evidence indicates that the cells in XX and XY gonads are bipotential and capable of differentiating along either the male or female pathway (Koopman *et al.*, 1990; Palmer and Burgoyne, 1991a; Albrecht *et al.*, 2001). Expression of *Sry* in the XY gonadal primordium causes the somatic supporting cell precursors of the gonad to differentiate as Sertoli cells. In the absence of *Sry*, the somatic supporting cell precursors follow the ovarian pathway and eventually become follicle cells.

The development of PGCs into either sperm or oocytes depends upon the environment in which they reside (for a review, see McLaren, 1995). Regardless of their chromosomal makeup (XX or XY), PGCs synchronously enter meiosis by 13.5 dpc in the absence of any interference from Sertoli cells. In the ovary, PGCs arrest in the first prophase of meiosis I until later ovulatory cycles (McLaren and Southee, 1997; McLaren, 2000). Many experiments have shown that PGCs are required for the differentiation of follicle cells; in the absence of PGCs, follicles never form (Huang *et al.*, 1993; Bedell *et al.*, 1995). In contrast, the differentiation of Sertoli cells and formation of testis cords are not dependent upon PGCs; in the absence of PGCs, testis cords form normally. The differentiation of Sertoli cells in the gonad leads to the enclosure of PGCs inside testis cords and their arrest in mitosis before 13.5 dpc (Kurohmaru *et al.*, 1992; McLaren and Southee, 1997).

Sertoli cells are believed to act as an organizing center for the testis by directing all other cell types into their respective lineages. Experiments with XX ↔ XY mosaic mice revealed that the only cell type with a strong bias for a Y chromosome is the Sertoli cell (Palmer and Burgoyne, 1991a). In mosaic gonads that became testes, 90% of Sertoli cells were XY and 10% were XX. All other somatic cell types displayed a 50/50 XX:XY distribution. From these results, it was concluded that *Sry*-expressing Sertoli cells are capable of initiating the differentiation of all other cell types within the testis. These experiments also revealed that *Sry* is not required cell autonomously for Sertoli differentiation, since 10% of Sertoli cells in these experiments were XX. It is likely that *Sry*-expressing somatic cells are capable of recruiting other non-*Sry*-expressing somatic cells into the Sertoli lineage. Sertoli cells – and probably follicle cell precursors – have been shown to arise from the coelomic epithelium before 11.5 dpc. This led us to hypothesize that coelomic epithelial cells would express *Sry* (Karl and Capel, 1998; Schmahl *et al.*, 2000). However, more recent experiments have localized *Sry* expression to a population of somatic cells that lies underneath

the coelomic epithelium at 11.2 to 11.5 dpc (Bullejos and Koopman, 2001; Albrecht *et al.*, 2001). This expression pattern suggests that pre-Sertoli cells turn on *Sry* and other genes required for Sertoli differentiation after they delaminate from the coelomic epithelium and enter the gonad. It is also possible that an additional population of Sertoli cells that express *Sry* arise from the mesonephros.

The Leydig cells of the XY gonad are required for production of testosterone, which initiates development of the male reproductive tract and male secondary sex characteristics. All available data suggest that these cells originate from the mesonephros before 11.5 dpc. *Steroidogenic factor 1 (Sf1)*, a gene expressed in steroid-producing cells of the adrenal and Leydig cells of the testis, labels cells in the anterior end of the mesonephros prior to 11.5 dpc. These cells contribute to the adrenal gland and the XY gonad and represent a likely source of Leydig precursors (Hatano *et al.*, 1996; Morohashi, 1997). In one electron microscopy study, Leydig cells were identified among a marked population of cells that had originated from the mesonephros (Merchant-Larios and Moreno-Mendoza, 1998). Finally, experiments in which XY gonads were separated from their mesonephroi at 11.5 dpc and cultured several days revealed that testosterone still was produced by these gonads. This indicated that Leydig precursors already are present in the gonad at 11.5 dpc (Merchant-Larios, 1979). The counterpart of the Leydig cell in the ovary is the theca cell. Precursors of theca cells are not identifiable early in the ovary but their origins are believed to be parallel to that of Leydig cells.

The other known somatic lineages in the XY gonad include peritubular myoid cells and endothelial cells. Myoid cells, a smooth muscle lineage, have been shown to collaborate with Sertoli cells in setting up the tubule structure of the testis (Skinner *et al.*, 1985). In the adult, they generate a peristaltic action to pump sperm through the tubules. Myoid cells have no analogous lineage in XX gonads. Endothelial cells are present in both XX and XY gonads; however, by 12.5 dpc, the vasculature of XY gonads undergoes major structural changes. The most notable of these vascular reorganizations is the formation of a large coelomic surface vessel.

IV. Genes Required for Testis and/or Ovary Development

Several genes have been identified that play roles in the early formation of the gonads in both sexes. These include *Sf1*, *Wilm's tumor (Wt1)*, *Lim1*, *Lhx9*, and *Emx2*. *Sf1* encodes a member of the nuclear hormone receptor family that is expressed in the somatic cells of XX and XY genital ridges as the gonad begins to form at 10.0 dpc (Ikeda *et al.*, 1994). Expression continues in the precursors of Leydig and Sertoli cells in XY gonads until 12.5 dpc, then gradually becomes restricted to Leydig cells. In XX gonads, *Sf1* expression can be seen in somatic

cells until 13.0 dpc (Ikeda *et al.*, 1996). *Sfl*^{-/-} mice initiate development of the gonadal primordium; however, by 12.5 dpc, the gonads of both XX and XY individuals regress by apoptosis (Luo *et al.*, 1994). The *Wtl* gene also is expressed in the somatic cells of the gonadal primordium of both sexes (Rackley *et al.*, 1993). In *Wtl*^{-/-} mice, neither kidneys nor gonads form (Kriedberg *et al.*, 1993). Recent *in vitro* assays using human forms of WT1 and a reporter driven by the *SRY* promoter suggest that WT1 is capable of upregulating *SRY* expression (Hossain and Saunders, 2001). *Lim1*, *Lhx9*, and *Emx2* also are involved in the early formation of the gonad in both sexes. *Lim1* and *Lhx9* are homeodomain proteins and *Emx2* encodes a homologue of a *Drosophila* head gap gene. Mice carrying null mutations for any of these genes do not form gonads (Shawlot and Behringer, 1995; Miyamoto *et al.*, 1997; Birk *et al.*, 2000). Studies have suggested that *Lhx9* may lie upstream of *Sfl*, since *Sfl* expression in *Lhx9* null mice is dramatically reduced (Birk *et al.*, 2000).

A number of genes are expressed specifically in the XY gonad after 11.5 dpc and have been identified as early players in the sex-determination cascade. *Sox9* is expressed in both XX and XY gonads beginning at 10.5 dpc but persists only in the nuclei of Sertoli cells of XY gonads after 11.5 dpc (daSilva *et al.*, 1996; Kent *et al.*, 1996). Humans heterozygous for mutations in *Sox9* display varying degrees of sex reversal (Cameron *et al.*, 1996). However, mice heterozygous for mutations in *Sox9* form normal testes. Mice homozygous for null mutations in *Sox9* have been generated but die before 11.5 dpc, preventing study of the gonads. Efforts are underway to generate chimaeric mice with *Sox9*^{+/+} and *-/-* cells, which may allow analysis of *Sox9* function during gonadogenesis (R. Behringer, personal communication). Experiments in which *Sox9* is ectopically expressed in XX gonads under the control of the *Wtl* promoter have shown that *Sox9* can cause sex reversal in the absence of *Sry* (Vidal *et al.*, in press). Recently, a transgene insertion upstream of the *Sox9* promoter, *odsex* (for ocular degeneration with sex reversal), was shown to result in misexpression of *Sox9* and sex reversal of XX gonads (Bishop *et al.*, 2000). Thus, activation of *Sox9* seems to account for all the effects of *Sry*. Integration of the transgene in *odsex* resulted in the deletion of sequences one to two megabases upstream of the *Sox9* coding region. From these results, it was proposed that the portion of DNA deleted in *odsex* mice is the binding site for a negative regulator of *Sox9*. In normal males, the presence of *Sry* antagonizes this regulator, allowing *Sox9* to be transcribed. However, in normal females, where *Sry* is not present, *Sox9* is silenced in the presence of the negative regulator. In the *odsex* deletion, removal of the binding site from a distant regulatory region would allow for the ectopic expression of *Sox9* in the XX gonad.

Müllerian inhibiting substance (MIS), a member of the transforming growth factor beta (TGF β) family, is expressed specifically in the Sertoli cells of the XY gonad, beginning between 11.5 and 12.5 dpc (Munsterberg and Lovell-Badge,

1991). At 11.5 dpc, the mesonephros contains two ducts, the Müllerian duct and the mesonephric duct, which are the precursors of the female and male reproductive tracts, respectively (Josso and Picard, 1986; Donahoe *et al.*, 1987). In females, the Müllerian duct develops in the absence of MIS, whereas in males, MIS production leads to the regression of this duct by apoptosis. Misexpression of MIS in the XX gonad leads to regression of the Müllerian duct, while in XY *Mis*^{-/-} mice, the Müllerian duct fails to regress and both male and female reproductive tracts develop (Behringer *et al.*, 1990,1994). The mesonephric, or Wolffian duct, develops into the male reproductive tract. Testosterone is required for the development of this duct. In the absence of testosterone in females, the mesonephric duct regresses.

Another gene expressed specifically in Sertoli cells beginning at 12.5 dpc is *Desert hedgehog (Dhh)*. XY *Dhh*^{-/-} mice are sterile (Bitgood *et al.*, 1996) and show defects in Leydig cell formation and Sertoli-myoid cell interactions in the adult testis (Clark *et al.*, 2000). *Patched1 (Ptc1)*, which encodes a receptor for *Dhh*, is expressed by numerous cells of the interstitium, including peritubular myoid cells and Leydig cells. *Dhh* and *Ptc1* are not known to be expressed by XX gonads at any stage of development.

Fgf9 is expressed specifically in XY gonads at 11.5 and 12.5 dpc. Analysis of *Fgf9*^{-/-} mice revealed that the majority of XY mice developed as phenotypic females (Colvin *et al.*, 2001). In these XY mice, 80% of the gonads developed as ovaries and 20% developed some abnormal testis cords. Analysis of proliferation in these mice after 12.5 dpc showed that proliferation rates for interstitial cells were less than 70% that of controls. Furthermore, culturing XX gonads in the presence of FGF9 resulted in the recruitment of mesonephric cells into the XX gonad. These data suggest that *Fgf9* plays an early role in regulating male-specific pathways downstream of *Sry*.

Another gene that, when deleted in mice, causes male-to-female sex reversal is *M33*. *M33* is a homologue of the *Drosophila polycomb* group genes (Kato-Fukui *et al.*, 1998). It is not clear where in the sex-determination hierarchy *M33* acts. Since both polycomb and HMG proteins have been shown to alter chromatin structure, there may be interactions between *M33* and *Sry* that regulate domains of gene expression.

DMRT1 was identified by its homology to genes with DNA-binding motifs termed DM domains. Two such genes are *mab-3* in *C. elegans* and *doublesex (dsx)* in *Drosophila*, genes that play roles in sex determination and male-specific differentiation in these organisms (Raymond *et al.*, 1998). The DMRT1 gene in humans maps to a cluster of DMRT genes in a region of chromosome 9 associated with defective testicular development. In mice, *Dmrt1* is expressed in the gonads of both sexes at the early stages of gonad development. By 13.5 to 14.5 dpc, it becomes specific to the testis (Raymond *et al.*, 1999). Testes in mice null for *Dmrt1* are normal at the early stages of testis development. At later stages

after birth, the seminiferous tubules of *Dmrt1*^{-/-} XY gonads begin to degenerate. Ovarian development is normal in *Dmrt1* null mice (Raymond *et al.*, 2000). While these results do not place *Dmrt1* early in the sex-determination pathway in mammals, it is possible that there is redundancy with other *Dmrt* family members.

Dax1 and *Wnt4* are genes expressed specifically in XX gonads after 11.5 dpc. *Dax1* was cloned from an X chromosomal region in humans responsible for dosage-sensitive sex reversal (DSS) (Bardoni *et al.*, 1994; Zanaria *et al.*, 1994). Studies by Swain *et al.* (1998) have suggested that *Dax1* plays a role in ovarian development by inhibiting *Sry* action. However, removing the *Dax1* gene from mice had no effect on ovary development but instead led to male sterility and Leydig cell hyperplasia (Yu *et al.*, 1998). Therefore, the function of *Dax1* during gonadogenesis is not entirely clear. *Wnt4*^{-/-} females exhibit ectopic Leydig cell development, whereas XY *Wnt4*^{-/-} mice have no obvious defects in testis development (Vanio *et al.*, 1999). These results suggest that *Wnt4* plays an inhibitory role in XX gonads by repressing a portion of the male pathway that leads to the differentiation of Leydig cells. In XY gonads, expression of *Sry* and *Sox9* somehow leads to the repression of *Wnt4*, allowing Leydig cell development to proceed. (See Table I for a list of genes involved in testis and ovary development.)

V. Genetic Models of Sex Determination

Analysis of the formation of ovotestes in C57BL/6 (B6) XY^{POS} mice has provided insight into the mechanisms governing the decision to become a testis or an ovary. When the Y^{POS} chromosome from *Mus musculus poschiavinus* is crossed onto a B6 background, XY^{POS} mice display a delay in testis cord formation that leads to varying degrees of sex reversal, ranging from the formation of ovotestes (gonads containing both testis and ovarian regions) to ovaries (Eicher *et al.*, 1982; Palmer and Burgoyne, 1991b). To account for the occurrence of ovotestes in B6XY^{POS} mice, it has been proposed that male-specific pathways must be initiated by 11.5 dpc in order to repress female-specific pathways; otherwise, ovarian development proceeds. Several studies have attempted to link the delay in testis cord formation to a delay in *Sry* expression, with inconsistent results (Nagamine *et al.*, 1999; Lee and Taketo, 2000). However, evidence from transgenic animals also supports this idea. Experiments attempting to rescue the defect in B6XY^{POS} mice with an *Sry* transgene are only successful if *Sry* is expressed at sufficient levels and in the proper temporal pattern (Eicher *et al.*, 1995). The Y^{POS} chromosome is capable of inducing normal testis formation on other genetic backgrounds. This has led to the hypothesis that incompatibilities exist between the Y^{POS} *Sry* gene and alleles of other genes that are specific to B6, termed TDAs (testis-determining autosomal genes). Mapping

TABLE I
Genes Involved in Mammalian Gonad Development

Gene	Expression	Function	Reference(s)
<i>Sfl</i>	M,F	Early establishment of gonads, regulator of genes for steroid production	Luo <i>et al.</i> , 1994; Ikeda <i>et al.</i> , 1994,1996
<i>Wt1</i>	M,F	Early establishment of gonads and kidneys, possible regulator of <i>Sry</i> expression	Rackley, 1993; Kriedberg <i>et al.</i> , 1993
<i>Lim1</i>	M,F	Early establishment of gonads	Shawlot and Behringer, 1995
<i>Emx2</i>	M,F	Early establishment of gonads	Miyamoto <i>et al.</i> , 1997
<i>Lhx9</i>	M,F	Early establishment of gonads	Birk <i>et al.</i> , 2000
<i>Sox9</i>	M	Sertoli cell differentiation, initiation of testis development	Cameron <i>et al.</i> , 1996; daSilva <i>et al.</i> , 1996; Kent <i>et al.</i> , 1996; Bishop <i>et al.</i> , 2000
<i>Mis</i>	M	Regression of Müllerian duct	Josso and Picard, 1986; Donahoe <i>et al.</i> , 1987; Behringer <i>et al.</i> , 1990,1994; Munsterberg and Lovell-Badge, 1991
<i>Dhh</i>	M	Regulator of Sertoli-myoid, Sertoli-Leydig interactions, development of male germ cells	Bitgood <i>et al.</i> , 1996; Clark <i>et al.</i> , 2000
<i>Fgf9</i>	M	Proliferation in male gonad	Colvin <i>et al.</i> , 2001
<i>M33</i>	Unknown	Required for testis development	Katoh-Fukui <i>et al.</i> , 1998
<i>Dmrt1</i>	M	Maintenance of seminiferous tubules after birth	Raymond <i>et al.</i> , 1998,1999,2000
<i>Dax1</i>	F	Unclear	Swain <i>et al.</i> , 1998; Yu <i>et al.</i> , 1998
<i>Wnt4</i>	F	Repression of Leydig cell differentiation in female gonads	Vanio <i>et al.</i> , 1999

studies have identified regions on chromosomes 2, 4, and 5 that are associated with the formation of ovotestes (Eicher *et al.*, 1996).

VI. Cellular Pathways Downstream of SRY

Despite the discovery of multiple genes involved in the testis pathway, no direct targets of SRY have been identified. In this 24-hour period, SRY estab-

lishes the expression of genes involved in the rapid patterning of the gonadal cells into testis cords and a male-specific pattern of vasculature. Virtually nothing is known about the genes that SRY activates to govern the cellular changes that occur in the XY gonad between 11.5 and 12.5 dpc. An alternative approach is to characterize the cellular pathways initiated by SRY. Knowledge of these pathways and how they operate in testis formation can be used to identify genes required for these processes and to link them to SRY.

Experiments have revealed several cellular pathways downstream of *Sry* that play a role in testis formation. These include cell proliferation, commitment of coelomic epithelial cells to the Sertoli lineage, mesonephric cell migration, and vascularization. All of these pathways have been shown to be required for normal testis formation and depend upon the expression of *Sry* in the genital ridge.

A. PROLIFERATION OF SERTOLI PRECURSORS AND COMMITMENT TO THE SERTOLI LINEAGE

The Sertoli cell plays a central role in testis development by directing all other cell types into their respective lineages. For this reason, it is important to understand the origin of this cell type. Previous studies have suggested two sources of Sertoli cells: the coelomic epithelium and the mesonephros. By labeling cells of the coelomic epithelium with DiI, experiments have shown that, before 11.5 dpc, the cells of the coelomic epithelium contribute to the Sertoli lineage (Karl and Capel, 1998). Furthermore, prior to 11.5 dpc, the cells of the coelomic epithelium can give rise to daughter cells of two lineages: Sertoli cells and unidentified interstitial cell types. After 11.5 dpc, coelomic epithelial cells still contribute to the XY gonad but only become interstitial cells.

In agreement with DiI studies, experiments using bromodeoxyuridine (BrdU) to label dividing cells showed that the coelomic epithelium is highly proliferative prior to 12.0 dpc (Schmahl *et al.*, 2000). These experiments defined two stages of proliferation in the XY gonad. The first stage of proliferation occurred in a population of cells that expresses high levels of *Sfl* and contributes to the Sertoli lineage as well as an interstitial lineage. A second stage of proliferation occurred at the coelomic epithelium after 11.5 dpc in a population of cells that expressed *Sfl* at low levels and contributed to interstitial lineages. To determine if proliferation was dependent upon *Sry* expression, the proliferation pattern of XX gonads from mice carrying an *Sry* transgene was examined and found to be identical to that of XY gonads. Conversely, XY gonads from B6XY^{pos} mice, which become ovaries or ovotestes, displayed levels of proliferation similar to XX gonads.

From these data, it was concluded that *Sry* induces the upregulation of proliferation in cells at the coelomic epithelium. Recent expression studies have shown that *Sry* is not expressed in coelomic epithelial cells (Bullejos and

Koopman, 2001; Albrecht *et al.*, submitted). Therefore, *Sry*-expressing Sertoli precursors beneath the coelomic epithelium are likely to expand their population in a noncell-autonomous manner by recruiting non-*Sry*-expressing cells at the coelomic epithelium into the Sertoli lineage. Prior to 11.5 dpc, cell divisions at the coelomic epithelium result in daughter cells that follow different fates, as revealed in the *DiI* injection experiments. This might occur through the asymmetric distribution of cellular components during the division process, by lateral signaling between daughter cells, or through interactions with other somatic cells of the gonad once the division is complete. After 11.5 dpc, proliferation at the coelomic epithelium results in an expansion of the population of interstitial cells (for a diagram of male-specific proliferation, see Figure 1).

B. MESONEPHRIC CELL MIGRATION

Early electron microscopy studies on the structure of the gonad revealed similarities in morphology and cell staining between mesonephric and gonadal cells, suggesting that gonadal cells may have origins in the mesonephros. Experiments demonstrated that mesonephric cells contribute to the XY gonad and that culture of XY gonads without their adjoining mesonephroi resulted in a failure of testis cord formation (Buehr *et al.*, 1993; Merchant-Larios *et al.*, 1993). More recent experiments showed that cells migrated into the XY gonad between 11.5 dpc and at least 16.5 dpc and that this event did not occur in XX gonads. Migrating cells are represented in at least three lineages: peritubular myoid cells, endothelial cells, and cells associated with the endothelium (Martineau *et al.*, 1997). To determine whether cell migration directly depends upon *Sry* expression, this event was examined in XY mice lacking *Sry* and XX mice carrying *Sry* as a transgene. These experiments showed that cell migration did not occur in XY gonads destined to form ovaries. In contrast, XX gonads carrying an *Sry* transgene, which always formed testes, did recruit cells from the mesonephros (Capel *et al.*, 1999). Furthermore, XY gonads with weak alleles of *Sry* that result in ovotestes recruited cells only into testicular regions of the gonad (Albrecht *et al.*, 2000). These experiments showed that cell migration from the adjacent mesonephros is one of the earliest events induced by *Sry* and strongly suggested a role for cell migration in the formation of testis cords.

Initial attempts to characterize the signal(s) for cell migration demonstrated that culturing an XX gonad with an XY gonad at its coelomic surface resulted in migration of mesonephric cells into the XX tissue (Martineau *et al.*, 1997). Likewise, culture of XX gonads with beads coated with proteins from XY gonads led to the induction of cell migration. This assay was used to further investigate the role of cell migration in the developing gonad. Examination of the structure of XX gonads that had been cultured with an XY gonad at their coelomic surface

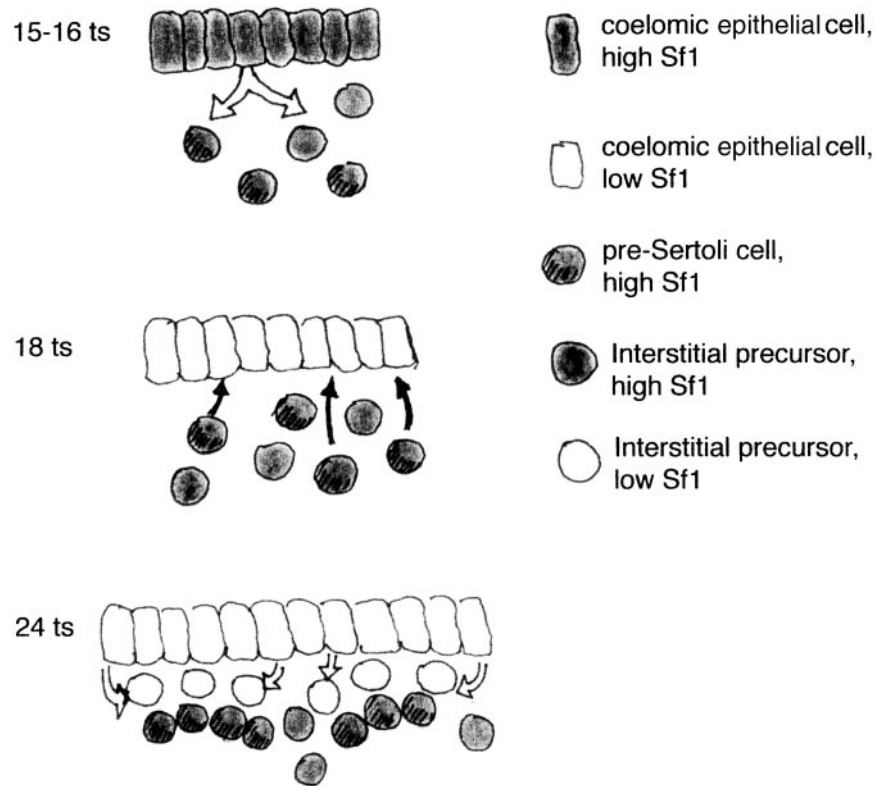


FIG. 1. Model for stages of male-specific proliferation in the mammalian gonad. At 15–16 tail somites (ts), corresponding to 11.25 dpc, cells of the coelomic epithelium, expressing Sf1 at high levels, divide (outlined arrow) and give rise to cells destined to become Sertoli cells as well as interstitial cells. At 18 ts, corresponding to 11.5 dpc, pre-Sertoli cells, expressing high levels of Sf1, signal to cells at the coelomic epithelium (solid arrows), expressing Sf1 at low levels, to continue dividing. At 24 ts, corresponding to 12.0 dpc, cells at the coelomic epithelium give rise (outlined arrows) to cells of interstitial lineages.

as a “sandwich” to induce mesonephric cell migration revealed that the XX gonadal cells organized into structures similar to testis cords. Laminin was deposited around cord structures and germ cells were enclosed within these cords. Furthermore, induced XX gonads upregulated the expression of the male-specific gene *Sox9* and downregulated the female-specific gene *Dax1*. These experiments showed that mesonephric cell migration is capable of inducing Sertoli cell differentiation in the absence of *Sry* in the XX supporting cell precursors (Tilman and Capel, 1999). These results suggested that interactions between migrating cells and supporting cell precursors play an important role in

the establishment of the Sertoli fate (for a diagram of male-specific cell migration, see Figure 2).

C. TESTIS DEVELOPMENT MUST BE INITIATED WITHIN A NARROW WINDOW

“Sandwich” experiments also defined a window in which cord formation could be induced in the XX gonad. While 11.5 dpc XX gonads were capable of organizing cords and expressing male-specific genes, 12.5 dpc XX gonads were not. This timeframe is consistent with the models proposed based on ovotestis formation in B6XY^{pos} mice. Interestingly, the window in which cords could be induced to form in “sandwich” experiments coincides with the timing of PGC entry into meiosis. Previous experiments showed that PGCs enter meiosis by 13.5 dpc, unless enclosed within testis cords (McLaren and Southee, 1997). Because it is known that PGCs are required for the initiation of follicle formation (Huang *et al.*, 1993; Bedell *et al.*, 1995), it is likely that important interactions are

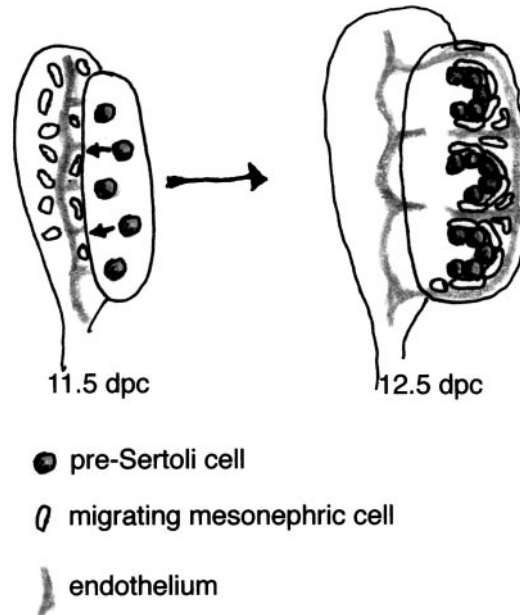


FIG. 2. Model for male-specific cell migration in the mammalian gonad. At 11.5 dpc, pre-Sertoli cells of the XY gonad (solid arrows) signal endothelial cells and other cells in the mesonephros to begin migrating into the gonad. By 12.5 dpc, interactions between migrating mesonephric cells and pre-Sertoli cells establish testis cords. Migrating cells are represented in at least three lineages: peritubular myoid cells, endothelial cells, and cells associated with the endothelium.

established between the somatic and germ cells of the XX gonad by 12.5 dpc that antagonize male-specific pathways. Several characterized cases of XX sex reversal result in the formation of tubules in ovaries older than 12.5 dpc and all are associated with the elimination of meiotic germ cells from the ovary (Vigier *et al.*, 1987; Merchant-Larios and Taketo, 1991; Whitworth *et al.*, 1996). These data support a model in which male-specific pathways must be initiated prior to 12.5 dpc in order to prevent PGCs from entering meiosis, an event that antagonizes testis formation by initiating follicle cell differentiation.

D. VASCULARIZATION

One of the most noticeable structures that appears in the XY gonad by 12.5 dpc is a large vessel just under the coelomic epithelium, termed the coelomic vessel. Early experiments designed to study cell migration into the XY gonad revealed that endothelial and associated cells enter the XY gonad from the mesonephros beginning at 11.5 dpc (Martineau *et al.*, 1997). Cell migration occurs until at least 16.5 dpc, specifically in XY gonads. Many of the migrating endothelial cells become part of the coelomic vessel as well as vessels in the interstitium of the testis. The dimorphic development of the vasculature in XX and XY gonads led to the hypothesis that construction of a male-specific vasculature plays an early role in the patterning and function of the testis.

To begin to address the role of migrating endothelial cells in testis formation, the mechanisms for vascular development in XX and XY gonads were compared and contrasted (Brennan *et al.*, submitted). At 11.5 dpc, the pattern of vessels appears similar between XX and XY gonads, as revealed by staining of gonads from Tie-2::LacZ mice, which contain an endothelial-specific LacZ transgene. However, by 12.5 dpc, the XY vasculature undergoes rapid reorganization that does not occur in XX gonads. The most noticeable features of the reorganization are the formation of the coelomic vessel, restriction of endothelial cells to the interstitial space between the testis cords, and a generation of a more highly branched system of vessels. The vasculature of the XX gonad expands during this period but its pattern does not change.

Cell migration from the mesonephros into the XY gonad is certainly one mechanism that accounts for this rapid reorganization and growth of the XY gonad. To determine whether endothelial migration from the mesonephros represented a specific subset of the vasculature (e.g., lymphatics, arteries, veins), the expression patterns of markers specific to these populations were examined in XX and XY gonads. Lymphatics did not populate the XX or XY gonad until 17.5 dpc. Examination of the arterial and venous specific markers, Ephrin B2 (arterial) and its receptor EphB4 (venous), revealed that, at 11.5 dpc, endothelial cells of XX and XY gonads express both arterial and venous markers in similar patterns, suggesting that these two populations have not been distinctly specified.

Interestingly, by 12.5 dpc, the vasculature of the XY gonad labels primarily with the arterial specific marker, Ephrin B2. In contrast, the vasculature of the XX gonad at 12.5 dpc continues to express both arterial and venous markers, suggesting that it has not been specified into distinct populations.

From this analysis, it was concluded that the *Sry* is required for the recruitment of endothelial cells from the mesonephros. These cells are required for the formation of the coelomic vessel and the respecification of the XY vasculature into an arterial system, which results in the rerouting of blood flow, primarily through the coelomic vessel. One explanation for this pattern of specification and blood flow in the XY gonad is that this event is necessary for the rapid export of testosterone from the testis, which occurs by 13.5 dpc. Future experiments will address the role of genes involved in the specification of the XY vasculature.

VII. Summary

Defects in sexual development are among the most common birth defects in humans. Since these defects can be a result of early events in the decision to form testes or ovaries occurring improperly, it is important to identify genes operating at these early steps. Identification of the cellular pathways that operate downstream of *Sry* has provided insight into the mechanisms of testis formation and will provide a foundation for identification of the genes involved in these pathways. Since SOX9 seems to account for all the effects of SRY, it is likely that activation of male-specific genes involved in proliferation, cell migration, and vascularization will depend upon SOX9. These genes are likely to be secreted signals and surface molecules involved in the proper sorting and specification of cells within the XY gonad. These genes must be downregulated in the XX gonad. This is likely to occur through the expression of molecules such as *Wnt4* and *Dax1*.

Differential expression studies have begun to identify genes expressed in both XX and XY gonads that may play roles in testis or ovary development. One such gene is *vanin-1*, which is expressed specifically in the Sertoli cells of the XY gonad (Bowles *et al.*, 2000). Serendipitous phenotypes in both XX and XY null mice (e.g., *Fgf9*, *M33*, *Wnt4*) have helped uncover several genes involved in gonad development as well. Mutagenesis screens are underway to identify new genes that influence testis or ovary development (R. Lovell-Badge, personal communication).

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