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Minireview: Transcriptional Regulation of Gonadal Development and Differentiation

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The embryonic gonad is undifferentiated in males and females until a critical stage when the sex chromosomes dictate its development as a testis or ovary. This binary developmental process provides a unique opportunity to delineate the molecular pathways that lead to distinctly different tissues. The testis comprises three main cell types: Sertoli cells, Leydig cells, and germ cells. The Sertoli cells and germ cells reside in seminiferous tubules where spermatogenesis occurs. The Leydig cells populate the interstitial compartment and produce testosterone. The ovary also comprises three main cell types: granulosa cells, theca cells, and oocytes. The oocytes are surrounded by granulosa and theca cells in follicles that grow and differentiate during characteristic reproductive cycles. In this review, we summarize the molecular pathways that regulate the distinct differentiation of these cell types in the developing testis and ovary. In particular, we focus on the transcription factors that initiate these cascades. Although most of the early insights into the sex determination pathway were based on human mutations, targeted mutagenesis in mouse models has revealed key roles for genes not anticipated to regulate gonadal development. Defining these molecular pathways provides the foundation for understanding this critical developmental event and provides new insight into the causes of gonadal dysgenesis. (*Endocrinology* 146: 1035–1042, 2005)

LIKE MOST ORGAN systems, mammalian gonadal development involves a complex interplay of multiple cell types, and it occurs during a relatively narrow time window. Thus, it is important to understand the temporal pattern of gene expression as well as the spatial relationships of the developing tissues. For these reasons, most recent studies have used the mouse as a model for examining gonadal development because it is amenable to genetic manipulation as well as detailed histologic and biochemical characterization. During the transition from an undifferentiated gonad to a testis or ovary, the female and male gonad each display characteristic morphological features and patterns of gene expression (1). The coordinated differentiation of the embryonic urogenital ridge, from the bipotential state to sexual dimorphism, provides an opportunity to identify the key factors and commitment steps that underlie gonadal differentiation (2).

In humans, the functional significance of some of these molecules is evident from mutations that impair gonadal development and reproduction (3–5). For example, deletions of the Y chromosomal gene, *SRY* (sex-determining region on the Y chromosome) cause XY male-to-female sex reversal, whereas *SRY* translocations to the X chromosome lead to XX female-to-male sex reversal (6). The role of *Sry* as a candidate testis-determining gene was confirmed by demonstrating testis development after transgenic expression of *Sry* in XX mice (7). Thus, by using animal models, it is possible to explore genetic pathways in greater detail and elucidate disease pathogenesis.

Many of the gonadal development factors described to date are known to act at the transcriptional level. For the most part, their functions are incompletely understood. Based on homology to other transcription factors, some affect DNA bending (8) or modulate chromatin remodeling (9). Others form interactive complexes that activate transcription (10) or have a role in specifying progenitor cell types (11). Inhibition of gene expression is equally likely to be important as a means to dictate cell fate, but less is known about potential transcriptional repressors. The functional interaction between extracellular ligand molecules and nuclear transcription factors also merits emphasis. For example, extracellular signals can induce transcription factor release and translocation to the nucleus [*i.e.* β-catenin/lymphoid-enhancing factor, Smad (a name combining small and mothers against decapentaplegic), Janus kinase/signal transducer and activator of transcription, nuclear factor-κB, nuclear factor of activated T cells] (12). Increasing lines of evidence suggest that cross talk among intracellular signaling pathways mediate downstream transcriptional responses. Thus, it is important to identify the extracellular ligands, membrane receptors, and signal transduction pathways associated with gonadal development, as well as the transcription factors.

To date, numerous candidate genes have been identified based on their pattern of expression in the embryonic gonad. Gonadal phenotypes are increasingly recognized in knockout models originally designed to explore gene function in

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Abbreviations: AMH, Anti-Müllerian hormone; *Arx*, Aristaless-related homeobox; BMP, bone morphogenetic protein; *Dax1* dosage-sensitive sex reversal, adrenal hypoplasia congenita, X chromosome; *Dhh*, desert hedgehog; dpc, days post coitum; FGF, fibroblast growth factor; *Fst*, follistatin; HMG, high-mobility group; KTS, lysine-threonine-serine; MIS, Müllerian inhibitory substance; *Pdgfr-α*, platelet-derived growth factor receptor-α; PMDS, persistent Müllerian duct syndrome; *Pod1*, podocyte; *Sf1*, steroidogenic factor 1; *Sox9*, Sry related, HMG box; *Sry*, sex-determining region on the Y chromosome; *Wt1*, Wilms' tumor-1.

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other tissues. In many cases, embryonic lethality precludes analysis of adult reproductive function. Hence, for gene mutations that have pleiotropic effects, the gonadal phenotype must be studied primarily during embryogenesis, or by cell type-specific removal of a genetic locus. Further identification of novel candidate genes will rely on a combination of molecular and genetic tools, including forward and reverse genetics approaches.

A model for gonadal development postulates a series of sequential commitment steps as specific cell types achieve their final differentiated state. A goal in the field is to identify the genetic cascade that programs these events. Some of these steps are cell autonomous, meaning that a cell will pursue a particular fate, relatively independent of its environment. More often, key developmental steps occur via cell-to-cell communication, reflecting direct cellular contacts or responses to paracrine signals. Markers of differentiation include morphological changes, as well as alterations in gene or protein expression. A future challenge is to identify specific targets of key genes in the developmental cascade and to characterize the biochemical events associated with differentiation.

Organogenesis in the Urogenital Ridge

After gastrulation, organogenesis in the mouse embryo starts approximately 8 d post coitum (dpc). The gonad, adrenal gland, kidney, and reproductive tract can be traced to a derivative of the intermediate mesoderm—the urogenital ridge—that forms at embryonic d 9.5. At 10.5 dpc, the genital ridge thickens bilaterally on either side of the dorsal aorta and constitutes an outgrowth of epithelial cells that proliferate at the center of the coelomic cavity and extend along the anterior-posterior axis from the forelimb bud to the hindlimb bud. The urogenital ridge is tethered to the peritoneal cavity by mesentery and comprises two juxtaposed tissues: the gonad rudiment is located medially and the mesonephros, which contains dual Wolffian and Müllerian ducts, is more dorsal (Fig. 1, A and B). The metanephric kidney is an outgrowth of the caudal mesonephros and is visible by 12.5 dpc (Fig. 1B). The adreno-gonad primordium arises between the gonad and mesonephros on the dorsal side and buds anteriorly at 12.5 dpc to form the adrenal gland. In both sexes, the adrenal gland and kidney ascend toward the abdominal region. In contrast, the final position of the gonad depends on its differentiation into an ovary or testis, and on the interdependent maturation of the sex-specific Müllerian or Wolffian ducts. In the male, owing the action of testosterone, the proximal Wolffian duct is virilized to form the epididymis, vas deferens, and seminal vesicle. Regression of the distal Müllerian duct is mediated by anti-Müllerian hormone (AMH, or Müllerian inhibitory substance, MIS) (13). Another peptide hormone, insulin-like 3 (Insl-3) mediates testicular descent (13, 14). Persistent Müllerian duct syndrome (PMDS) in males is caused by loss of MIS, MIS type I, or type II receptor function (15–17). The absence of testosterone and MIS in females allows regression of the Wolffian duct and development of the Müllerian duct into oviduct, uterus, and upper vagina.

Genetic Program of the Bipotential Gonad

The gonad primordium is morphologically indistinguishable from 10.0 through 11.5 dpc in males and females. The testis cords become visible by 12.0 dpc and reflect the coalescence of Sertoli and germ cells that are surrounded by a layer of thin peritubular myoid cells. In males, the gonad begins to enlarge visibly at about 13.5 dpc, as a consequence of proliferation of germ cells and precursor somatic cells. The ovary, on the other hand, is seemingly more quiescent during this time, but female-specific markers provide evidence that ovarian differentiation is underway (18, 19).

Before expression of the male determining gene, *Sry* at 10.5 dpc, a number of factors act in concert to specify the urogenital ridge in both sexes (Fig. 2). Moreover, because the urogenital ridge is the primordium for the gonad, adrenal, kidney, and reproductive tract, multiple organs are often affected by mutations of these genes.

Wt1 (Wilms' tumor-1) encodes a zinc finger transcription factor originally identified as a tumor suppressor gene. Loss-of-function mutations cause three distinct pediatric syndromes that display urogenital defects affecting male development: WAGR (Wilms' tumor, aniridia, genitourinary abnormality, mental retardation), Denys-Drash syndrome, and Frasier syndrome. The *Wt1* homozygous knockout animal does not form kidney, adrenal, or gonad (20, 21). In the gonad, the expression of *Wt1* at embryonic d 9.5 suggests a role in specifying the coelomic epithelial cells in the devel-

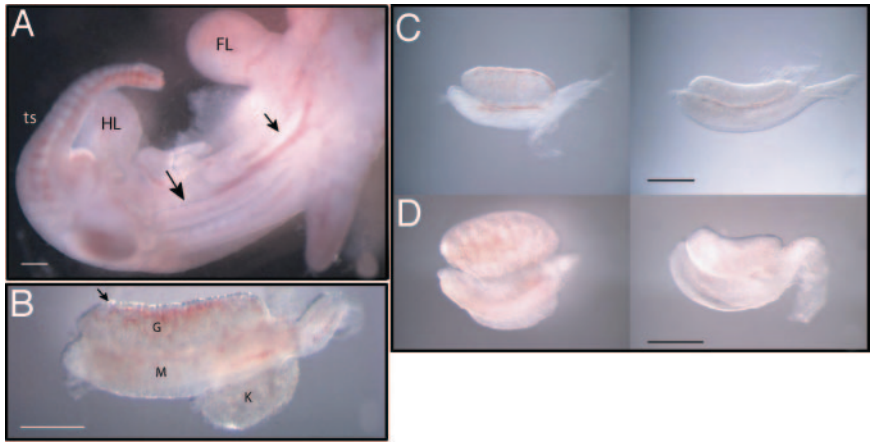

FIG. 1. At 11.5 dpc (A), the bipotential urogenital ridge (*large arrow*) flanks the dorsal aorta (*small arrow*) along the anterior-posterior axis in between the forelimb bud (FL) and hindlimb bud (HL). The gonad is closer to the midline, and the mesonephros develops dorso-lateral to the gonad. At 11.5 dpc, there are approximately 18 tail somites (ts) from the tip of the tail to the posterior end of the hindlimb bud. B, At 12.5 dpc, the male gonad shows distinct morphologic features including a prominent coelomic blood vessel (*arrow*). G, Gonad; M, mesonephros; K, kidney. C, By 13.5 dpc, the male gonad (*left*) is approximately twice the size of the female (*right*) due to increased cellular proliferation. D, Sexual dimorphism between the male (*left*) and female (*right*) gonad is further evident by 14.5 dpc (*scale bars*, 500 μm).

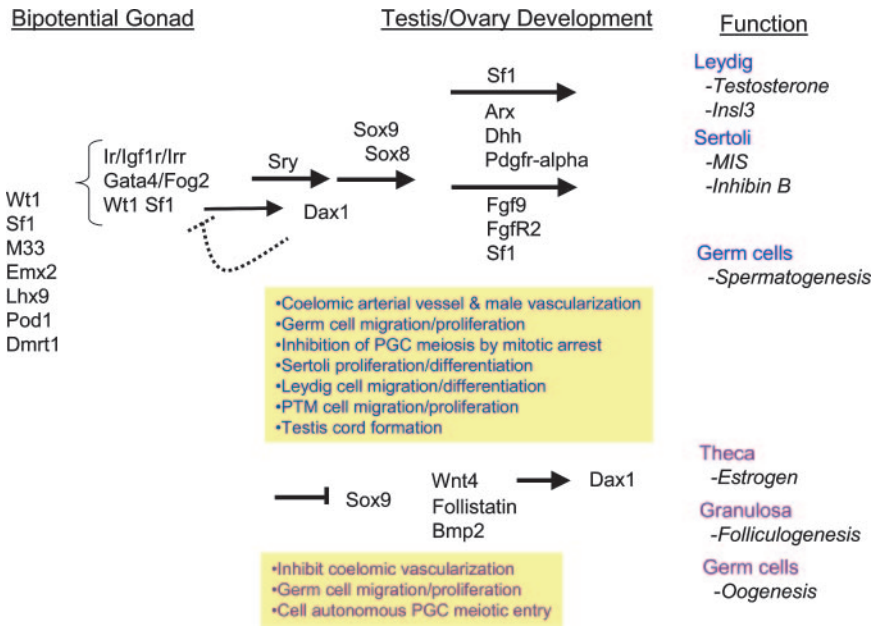

FIG. 2. Summary of genetic pathways involved in gonadal development. The bipotential gonad expresses genes such as *Wt1*, *Sf1*, *Emx2*, *M33*, *Lhx9*, *Pod1*, and *Dmrt1*. Subsequently, expression of the insulin receptor family *Ir*, *Irr*, and *Igf1r*; *GATA4/Fog2*; *Wt1*; and *Sf1* are thought to promote the expression of *Sry* in the male. *Sox9* and *Sox8* are downstream of *Sry*. *Sf1* regulates *Amh* activation in Sertoli cells and steroidogenesis in Leydig cells. In the male, *Dax1* may repress *Sf1* in a dose-dependent manner, modulating the transactivation of *Amh* and genes necessary for steroid hormone biosynthesis. *Dax1* also supports the normal formation of testis cords. Additional factors such as *Arx*, *Dhh*, *Pdgfr-α*, *Fgf9*, and *FgfR2* are involved in Leydig and Sertoli cell differentiation. A description of the cellular events downstream of *Sry* and thus specific to male development is outlined in the *center box*. PGC, Primordial germ cell; PTM, peritubular myoid cell. Female differentiation requires the activity of *Wnt4a* and repression of *Sox9*. Other key factors include *Fst*, *Bmp2*, and *Dax1*.

oping urogenital ridge and ensuring their survival (22). Two major isoforms have relevance to the developing urogenital organs: one alternatively spliced variant contains amino acids KTS (lysine-threonine-serine) (+KTS isoform), and the other lacks KTS (−KTS isoform). The additional amino acids lend flexibility to the protein between the third and fourth zinc finger and thereby diminish its DNA binding property. Both isoforms appear to have nonredundant functions in kidney and gonad development but not in the adrenal (23). In animals with genetically engineered ablation of the −KTS isoform, apoptosis was observed in streak gonads. *Sry* expression is reduced in the gonads of animals lacking the +KTS splice variant. Loss of either splice variant impairs testis descent.

Wt1 functions upstream of two orphan nuclear receptors *Sf1* (steroidogenic factor 1) and *Dax1* (dosage-sensitive sex reversal, adrenal hypoplasia congenita, X chromosome) (11). *Wt1* and *Sf1* synergize to enhance transcription of *Amh*, whereas *in vitro* experiments demonstrate this interaction is repressed by *Dax1* (10). *Sf1* also regulates the expression of multiple genes involved in male differentiation, steroidogenesis, and reproduction through *Sf1*-responsive DNA elements in target gene promoters [Ref. 24; and see review in this issue by Hammer *et al.* (25)]. *Sf1* stimulates *Dax1* transcription, and the two receptors are coexpressed in the adrenal, gonad, pituitary gonadotropes, and ventromedial hypothalamus (26). *Dax1* interacts directly with *Sf1* and functions as a transcriptional repressor of *Sf1*-regulated genes (27). In *Sf1* null mutants, neither XX nor XY animals form the adrenal or gonad. In the absence of *Sf1*, cells in both tissue rudiments undergo apoptosis, indicating that *Sf1* is necessary for survival of early progenitors of the adrenal and gonad (28). At 11.5 dpc, *Sf1* colocalizes with proliferating cells, suggesting that it directly or indirectly stimulates cell proliferation (29). *Dax1*-null males possess gonadal defects in testis cord morphogenesis, peritubular myoid cell proliferation, and spermatogenesis (30, 31). Hence, *Sf1* and *Dax1* are independently important for normal male gonadal differentiation.

Genes that primarily affect male gonadal differentiation or, have a male-specific expression pattern before distinct morphological changes, continue to expand the network of molecular pathways involved in testis development. Autosomal recessive male-to-female sex reversal (XY male bearing female gonads and feminized external genitalia) has been described in several mouse knockout models. *M33* (polycomb homolog) is involved in chromatin remodeling, yet its precise molecular role in gonadal differentiation is unknown (9). The LIM-domain containing transcription factor *Lhx9* (Lim homeobox) is required for full *Sf1* expression (11, 32). In contrast to the *Sf1* null animal, lack of testis development was not attributed to apoptosis in either *Lhx9* or *M33* mutant mice. *Pod1* (podocyte) is a basic helix-loop-helix transcription factor with a primary role in glomerular visceral epithelial cell (podocyte) differentiation and branching morphogenesis in the kidney and lung (33). In *Pod1* knockout mice, the gonads of both sexes are hypoplastic (34). Expression of *Pod1* in the male gonad was found mainly in the interstitium: peritubular myoid cells, pericytes associated with endothelium, and fetal Leydig cells. Defective coelomic vessel formation and attenuation of migrating endothelial cells from the mesonephros compromised vascularization in *Pod1* knockout male gonads. Although Sertoli development was not affected, there appeared to be expansion of the Sf1-positive Leydig cell population. *Dmrt1* (*doublesex*- and *mab*-related transcription factor) is homologous to genes in *Drosophila* and *Caenorhabditis elegans*, and null mutation causes dysmorphic testes in mice (35). *Maestro (Mro)*, *protease nexin-1* (*Pn-1*), and *vanin-1 (Vn-1)* were identified based on a sexually dimorphic expression pattern specific to the XY bipotential gonad (36–38). Later in development, *maestro* remains expressed in Sertoli and XY germ cells, whereas *protease nexin-1* and *vanin-1* are Sertoli restricted. Whether any of these newly described factors specify precursor populations, analogous

to the role of *Sry*-expressing Sertoli precursor cells, remains to be tested. The extent to which function can be attributed to a gene product relies on the phenotype of null mutation models in which are affected on all tissues where the gene of interest is expressed. Only recently have tissue-specific mutagenesis studies, for example with *Sf1* and *Sox9* [Sry related, HMG (high-mobility group) box], begun to elucidate spatial requirements attributed to developmentally regulated genes (39–41).

Supporting Cells: The Embryonic Testis Cord

The Sertoli cell is positioned at the base of the testis cord creating the perimeter of the tubule. Closely apposed is the elongated cell layer of peritubular myoid cells circumferentially surrounding the nascent cord. Testis cord morphogenesis is apparent in the male as early as embryonic d 12.0. The testis cords pattern the developing gonad and are the embryologic foundation for the adult seminiferous tubule. Sertoli precursor cells were identified at the coelomic surface (ventro-medial aspect) using dye tracking experiments that labeled dividing cells (42). Sertoli cells and granulosa cells may originate from the same precursor (43). In XX-XY chimeric studies, Sertoli cells were the only cell type that required *Sry* expression in a cell autonomous manner, whereas XX cells were preferentially excluded from the Sertoli population (44).

Sertoli cells, the cell lineage in which *Sry* is solely expressed in the male gonad, are thought to direct the differentiation of other cell types (45). For example, fetal Leydig cell differentiation depends on paracrine signaling through the extracellular protein Desert Hedgehog, which is secreted by Sertoli cells (46). Endothelial cells originating from the mesonephros are induced by a chemoattractant released by the male gonad (47). Two factors that can induce mesonephric cell migration are fibroblast growth factor (FGF) 9 and MIS, both of which are produced by Sertoli cells (48, 49). Sertoli cells also secrete a postulated factor(s) that induces mitotic arrest of germ cells (50). Unlike the female gonad, germ cells in the developing testis undergo mitotic arrest, being held as diploid progenitors until later in spermatogenesis. There is recent evidence that XY germ cells synthesize paracrine factors, including prostaglandin D_2, that induce Sertoli differentiation (51).

Sry expression occurs in a narrow temporal window from 10.5–12.0 dpc. The spatial pattern of *Sry* expression has been clarified by *in situ* hybridization analysis timed to tail somite stages. The central region of the gonad exhibits *Sry* expression first, followed by a rostral to caudal progression along the entire length of the gonad (52). Although *Sry* belongs to the HMG box containing family of nuclear transcription factors, evidence for downstream target genes remains elusive. A closely related gene, *Sox9*, is the other definitive male-determining gene identified thus far. Immunohistochemical labeling of genetically marked *Sry*-positive cells demonstrate that *Sry* and *Sox9* expression overlap in cells of the Sertoli lineage (53). As the *Sry* signal diminishes at 12.5 dpc, *Sox9* expression increases in the male and reaches a peak at 14.5 dpc. *Sox9*, which is weakly expressed in the undifferentiated gonad, is down-regulated in the female. Transgenic expression of *Sox9* on an XX genetic background is sufficient to cause female-to-male sex reversal (54). Heterozygous human *SOX9* mutations cause campomelic dysplasia, a severe skeletal disorder with defective cartilage development (55, 56). Many of these male patients also have gonadal dysgenesis. Heterozygous mice haploinsufficient for *Sox9* die perinatally due to skeletal malformations (57). An ingenious strategy was designed to transmit the knockout allele through the germline of otherwise wild-type male and female parents by the use of spermatocyte- and oocyte-specific promoters, respectively, enabling the zygote to obtain both knockout alleles (41). These experiments confirm that *Sox9* is necessary for male gonadal development. Notably, *Sox9* null embryos have elevated *Sry* expression, suggesting a negative feedback regulatory loop that down-regulates *Sry* in the mouse. Experimental evidence using mouse strains with a variety of *Sry* alleles supports the assertion that the precise timing, duration, threshold transcript levels, and protein isoform half-life, influence downstream events dependent on *Sry* expression (58, 59). *Sox8*, another member of the *Sox* HMG box containing transcription factor gene family, is also active in male development (60). It is possible that *Sry*, *Sox9*, and *Sox8* have partially redundant functions.

GATA4 and *Fog2* (friend of GATA) are important for cardiac morphogenesis but also influence gonadal development. A *GATA4* knock-in mutation, which prevents nuclear association of *GATA4* and *Fog2*, eliminates expression of the male differentiation markers *Sox9* and *Amh*. *Fog2* null mice have decreased *Sry* expression, loss of *Sox9*, *Amh*, and Desert hedgehog (*Dhh*), but persistence of the female marker *Wnt4* (61). The *GATA4* knock-in and *Fog2* null mice retain *Sf1* and *Wt1* expression, suggesting a hierarchical network. Similarly, *Ir, Irr, Igf1r* (insulin receptor, insulin-related receptor, Igf1 receptor) triple mutants have low *Sry* and *Sox9* expression and exhibit male-to-female sex reversal, thus implicating the insulin signaling pathway in male differentiation (62).

Anti-Müllerian hormone (AMH) or MIS is a key peptide hormone produced by Sertoli cells. It mediates the regression of the Müllerian duct, which would otherwise form female reproductive tract structures. In genetic males, PMDS is due to loss of MIS or cognate receptors present in mesenchymal cells of the Müllerian duct. PMDS is an example of male pseudohermaphroditism whereby males with testicular tissue appear feminized externally due to failure of testis descent caused by the presence of uterus and oviduct. Müllerian regression involves activation of a β-catenin-dependent pathway in a rostral-to-caudal manner (63). A number of factors are known to regulate *Amh* transactivation. These include *Sf1, WT1, GATA4, Sox9*, and *Sox8* (10, 64–66). MIS may have other functions that include mesonephric cell migration and vascularization in the male gonad, and germ cell loss in the ovary (49, 67).

Proliferation of pre-Sertoli cells is an important event in male development (29). By 13.5 dpc, the male gonad is twice the size of the female (see Fig. 1, C and D). One factor linked to proliferation is *Fgf9*. *Fgf9* null mice show varying degrees of male-to-female sex reversal (48). A number of Fgf receptors have been identified and *FgfR2* is thought to direct Sertoli cell differentiation perhaps as a result of nuclear translocation, which occurs simultaneously with translocation of

Sry and *Sox9* (68). A secreted protein, Fgf9, is a candidate male gonad-specific chemoattractant signal that induces migration of cells from the mesonephros into the gonad. Immigrant cells have been identified mainly as endothelial and peritubular myoid cells (47). Migration of these cells is necessary for testis cord morphogenesis (69) and induction of *Sox9* expression (70). This migration of mesonephric cells does not occur in females presumably because a chemoattractant is absent. Furthermore, once XX germ cells enter meiosis at 13.5 dpc, migration from the mesonephros is inhibited (71).

Hormone Biosynthesis: The Interstitial Space

Once sex determination has occurred, the ensuing phenotypic differentiation depends heavily on the production of steroid hormones. In the male, testosterone stabilizes the Wolffian duct and is converted by 5α-reductase to the potent derivative, dihydrotestosterone, which induces virilization of the male external genitalia. In the female, aromatase mediates estradiol synthesis. In some species, aromatase expression is critical for ovarian development; its role in ovary development in mammals is less clear, although aromatase deficiency is associated with transdifferentiation of granulosa cells into Sertoli-like cells (72).

Sf1 is restricted to Leydig cells after 13.5 dpc and mediates expression of several genes encoding enzymes required for testosterone biosynthesis including *StAR* (steroidogenic acute regulatory protein), *Cyp11a1*, *Cyp17* (cytochrome P450 hydroxylases), and *3βHSD* (hydroxysteroid dehydrogenase). The factors required for Leydig cell determination and lineage development are incompletely understood. Moreover, their origin, whether from immigrant mesonephric cells or progenitors within the gonad, remains equivocal. Leydig cell fate appears to rely on paracrine signals. *Dhh*, a Sertoli-secreted factor, induces expression of *Cyp11a1* (side-chain cleavage) in the Leydig cell (46). The *Dhh* signal is received by the receptor patched (*Ptc1*), which is expressed on Leydig cells. *Dhh* is thought to play a crucial role in Leydig cell differentiation and is an example of a paracrine signaling pathway identified between two developing cell types. Genetic analysis has placed *Pdgfr-α* (platelet-derived growth factor receptor-α) upstream of *Dhh* (73). Like Fgf9, Pdgfs induce mesonephric migration, and *Pdgfr-α* is necessary in the gonad for full *Cyp11a1* expression. The X-linked gene *Arx* (Aristaless-related homeobox) also influences Leydig cell development. However, it is expressed mainly in peritubular myoid cells, endothelial cells, and in the epithelium of the tunica albuginea (74).

Female Embryonic Gonadal Development

Relatively few genes have been shown to exhibit a female-specific pattern of gene expression early in gonadal development. To date, no gene has been shown to be a female-determining gene, as defined by conferring complete female-to-male sex reversal when mutated on an XX background, or by conferring male-to-female sex reversal after overexpression on an XY background. The *Dax1* gene was initially suggested as a pro-ovarian, or anti-testis, candidate gene because its duplication on an XY background is associated with impaired testis development (75, 76). However, *Dax1* loss of function on the XX background does not prevent ovary development (31). Subsequent studies have shown an unexpected role for *Dax1* in testis development (77), indicating that its actions are highly dependent on the timing and level of expression.

Although male somatic tissue can survive in the absence of germ cells, the female gonad depends on migratory germ cells to populate the undifferentiated primordium (78). In the female gonad, germ cells begin entering meiosis at 13.5 dpc and initiate a dynamic paracrine relationship with supporting cells destined to become cumulus and granulosa cells (79).

A growing list of genes exhibit an ovary-specific expression pattern. For example, *follistatin* (*Fst*) is highly expressed in the developing ovary relative to the testis (19). Follistatin binds members of the activin/TGFβ/bone morphogenetic protein (BMP) family. Thus, it may regulate the activity of one or more of these factors. *Stra8* (stimulated by retinoic acid) is also selectively expressed in the developing ovary and is thought to play a role in regulation of meiosis (80). Targeted mutagenesis and transgenic overexpression experiments will clarify the functions of these factors.

There has been postulated a "Z factor" that could act to suppress pro-testis events carried out by autosomal or X-linked genes in both XY and XX backgrounds (81). The Z factor would act downstream of *Sry* in genetic males. Therefore, the Z factor would be repressed by *Sry* in the male, and independent of *Sry* on an XX genetic background. Loss of a Z factor would be sex-reversing on XX (female-to-male), but gain-of-function on XY may or may not exhibit sex reversal (male-to-female) depending on whether the Z factor can override the downstream events of *Sry*. One candidate for the Z factor is *Wnt4* (Wingless-related integration site). Female *Wnt4* null mice develop virilizing gonads and Wolffian derivatives (82). This gonadal phenotype reflects the action of *Wnt4* to induce the migration of steroidogenic precursors of the adreno-gonad primordium into the anterior region of the gonad (83). Hence, misdirected adrenal steroidogenic cells become localized in the XX *Wnt4* knockout gonad. Secondly, *Wnt4* is down-regulated in males, whereas its expression remains strong in females where it prevents coelomic vascularization (a male feature, see Fig. 1B) through a molecular pathway that involves *Fst* and *Bmp2* (19). By inhibiting vascularization, *Wnt4* may prevent the proliferative growth expansion characteristic of male development. Thus, the homozygous knockout female gonad appears somewhat more male-like in shape and size. Externally, however, homozygous knockouts exhibit female genitalia. In humans, a loss-of-function mutation in *WNT4* caused Mayer-Rokitansky-Kuster-Hauser syndrome, which is characterized by defective development of Müllerian derivatives (84). Duplication of a chromosomal region containing *WNT4* was associated with a case of human XY sex reversal (85). However, overexpression of *Wnt4* on an XY background does not lead to XY sex reversal in the mouse (83, 86). Thus, the major roles of *Wnt4* in the female appear to include suppression of androgen-producing interstitial cells, inhibition of testis-like vascularization, and support of Müllerian derivatives. *Wnt4* is also essential for kidney (87), mammary gland (88), and

pituitary development (89), as well as normal functioning of the adrenal cortex (90).

A dominant insertional mutation led to identification of a female-to-male sex reversal phenotype in the *Odsex* (Ocular degeneration with sex reversal) mouse (91). The *Odsex* mouse was initially thought to lack a repressor for *Sox9*, allowing unabated *Sox9* expression ectopically in genetic females. Therefore, one of the functions of a Z factor could be to suppress *Sox9* action in the female gonad. However, it is also possible that regulatory elements contained in the inserted transgene promote activation of *Sox9*.

Nucleo-cytoplasmic shuttling of transcription factors may also provide an important regulatory step in sex determination. Organ culture experiments using the nuclear export blocker, leptomycin B, resulted in male-characteristic *Sox9* and *MIS* expression in XX gonad organ cultures (92). Thus, growth factors may be able to modulate transcriptional control by altering the location as well as the activity of various transcription factors.

Central Questions and Future Directions

The molecular pathways directing cellular differentiation can be traced back to cells of pluripotential origin. Thus, development must occur through a series of commitment steps driven by cell-to-cell contacts and paracrine interactions. In some cases, committed cells initiate a cell-autonomous program, as perhaps illustrated by *Sry* and *Sox9* actions in Sertoli cells. In other cases, differentiation is driven by secreted factors as illustrated by Dhh, Wnt4, and Fgf9. These cell autonomous and paracrine pathways are not mutually exclusive, and it is likely that most commitment steps integrate a series of internal and external signals. The sex determination pathway provides a unique paradigm for considering these events because the commitment steps lead to distinct cell types within a narrow time frame.

Despite impressive progress in this field, a number of challenging questions remain. What factors initiate *Sry* expression and what are its cellular targets? Are *Sry* and *Sox9* expression linked directly or do they reflect parallel, partially redundant pathways? Given that *Sox9* is sufficient to induce Sertoli cell development, what are its targets and how does it induce a network of genes they convey morphological changes characteristic of the testis? What are the genetic and molecular events that dictate ovary development? Although it is known that germ cells interact actively with somatic cells, what are the molecular and cellular events that control germ cell replication and entry into meiosis? How do meiotic germ cells foster ovary development? Implicit in these questions is the critical feature of timing during gonadal development. Once the Sertoli program is initiated, the developing testis induces mitotic arrest of germ cells. On the other hand, if Sertoli development is delayed, as occurs with various *Sry* alleles and mouse genetic backgrounds, germ cells begin to enter meiosis, and this step may further impair testis development, leading to mixed testis/ovary tissue or gonadal dysgenesis. Thus, future studies need to carefully assess pathways that control the timing and level of expression of regulatory factors.

Further understanding of these molecular and cellular functions in the gonad will provide examples of paradigms used to control cellular differentiation in other tissues. This knowledge will also be useful for the characterization of intersex cases and perhaps additional causes of idiopathic infertility. Parallel investigation of human mutations and animal models can accelerate progress in this field by identifying candidate genes and clarifying their roles in development.

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