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## The Transgenic Mouse in Studies of Mammalian Sexual Differentiation

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### INTRODUCTION

Throughout history, the subject of sex has held an inherent fascination. The musings of Aristotle on the role of “an infinitesimally minute but essential organ” in determining whether “the animal will in one case turn to male (or) in the other to female” (Aristotle, *Historia Animalium*), offer an early insight into what has become one of the tenets in our understanding of sexual differentiation in mammals: that the sex of the gonad determines the sexual development of the individual.

Sex determination in mammals is a remarkable process that has its origin in an indifferent gonadal primordium, common to both sexes, which has the ability to differentiate into either a testis or an ovary. In the presence of a Y chromosome, the indifferent gonad develops as a testis; in the absence of a Y chromosome, and regardless of the number of X chromosomes present, the indifferent gonad develops as an ovary. Remarkably, until fairly recently, this was about all we knew with respect to the genetic events involved in testicular differentiation. With the discovery in 1990 of *SRY* (sex-determining-region, Y chromosome gene), the testis-determining gene on the Y chromosome, the field seemed set for the systematic isolation of other genes functioning in the testicular differentiation pathway. Eleven years have now passed since the discovery of *SRY*, and the pathway from indifferent gonad to testis appears ever more complex. Our understanding of the ovarian differentiation process is even more limited.

The use of transgenic mice has contributed enormously to our understanding of the mammalian sex determination and sexual differentiation pathways, and hence is the

From: *Contemporary Endocrinology: Transgenics in Endocrinology*  
Edited by: M. Matzuk, C. W. Brown, and T. R. Kumar © Humana Press Inc., Totowa, NJ

focus of this chapter. While there is no denying the wealth of information gleaned from mouse models, it is also our belief that much can be learned from comparative studies of other mammalian systems. This chapter concludes with a brief introduction to the efforts that are underway to generate the first transgenic marsupial.

## SEX DETERMINATION: THE FATE OF THE GONAD

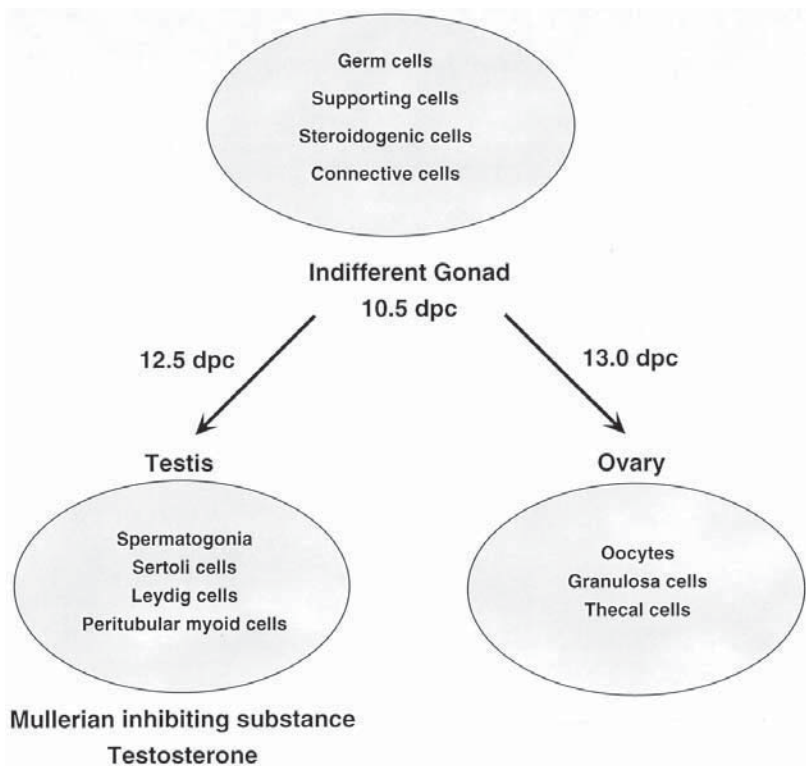
The mammalian testis and ovary have a common origin in the indifferent gonad. In the mouse, the indifferent gonad is first discernible at 10.5 d postcoitum (dpc) as a thickening on the medial aspect of the mesonephros. At this stage, the gonad consists of four known cell types—the primordial germ cells, supporting cells, steroidogenic cells, and connective cell lineage—each capable of entering the testicular or ovarian differentiation pathway (Fig. 1).

The indifferent male gonad of the mouse becomes sexually dimorphic at around 12.5 dpc when the supporting cell lineage gives rise to the Sertoli cells, which organize themselves into seminiferous cords that enclose the primordial germ cells. At the time that Sertoli cells form seminiferous cords, they begin secreting the glycoprotein Müllerian-inhibiting substance (MIS; also known as anti-Müllerian hormone, AMH). MIS is responsible for inducing the regression of the Müllerian ducts, which would otherwise persist to form the female reproductive tract. The Sertoli cells in turn appear to direct the differentiation of Leydig cells from the steroidogenic cell lineage, and peritubular myoid cells from the connective tissue (1). The production of testosterone by the Leydig cells stimulates the growth and virilization of the Wolffian ducts, which give rise to the epididymides, vasa deferentia, and seminal vesicles. At approx 13.5 dpc in the mouse testis, the germ cells become arrested in mitosis, marking their entry into the male pathway of development. Germ cells that fail to reach the gonad follow the female pathway and enter meiosis prematurely (2).

In the female gonad of the mouse, the germ cells enter meiosis at approx 13.0 dpc, becoming arrested at the first prophase 2 d later. From approx 16.5 dpc, granulosa cells—derived from the supporting cell lineage—surround the oocytes to form primordial follicles. Granulosa cells do not produce MIS until after birth, thus creating a permissive environment for the differentiation of the Müllerian ducts into the oviducts, uterus, and upper one-third of the vagina. Similarly, the fetal ovary does not produce testosterone, and consequently the Wolffian ducts degenerate.

Studies of XX↔XY chimaeric mice provided the first clue that the differentiation of the male supporting cells into Sertoli, rather than granulosa, cells is under the cell-autonomous control of a Y chromosome-encoded testis-determining factor (TDF in humans, *Tdy* in mouse) (1,3). In these mice, approx 95% of Sertoli cells are XY, and other testicular cell types are derived from XX and XY cells with equal probability. Thus, although the Y chromosome—and TDF/*Tdy*—is not essential for the differentiation of Leydig cells and peritubular myoid cells, it is required for Sertoli-cell differentiation.

The first example of a mutation to *Tdy* was described by Lovell-Badge and Robertson in 1990. Using XY embryonic stem (ES) cells that had been infected with a retroviral vector, they generated a male chimaera whose progeny included a small proportion of XY females. The retrovirally-induced mutation segregated with the Y chromosome. Further, during meiosis it was complemented by the *Sxr* and *Sxr'* translocations—both of which are translocations of the Y chromosome, including *Tdy*, onto the X chromo-

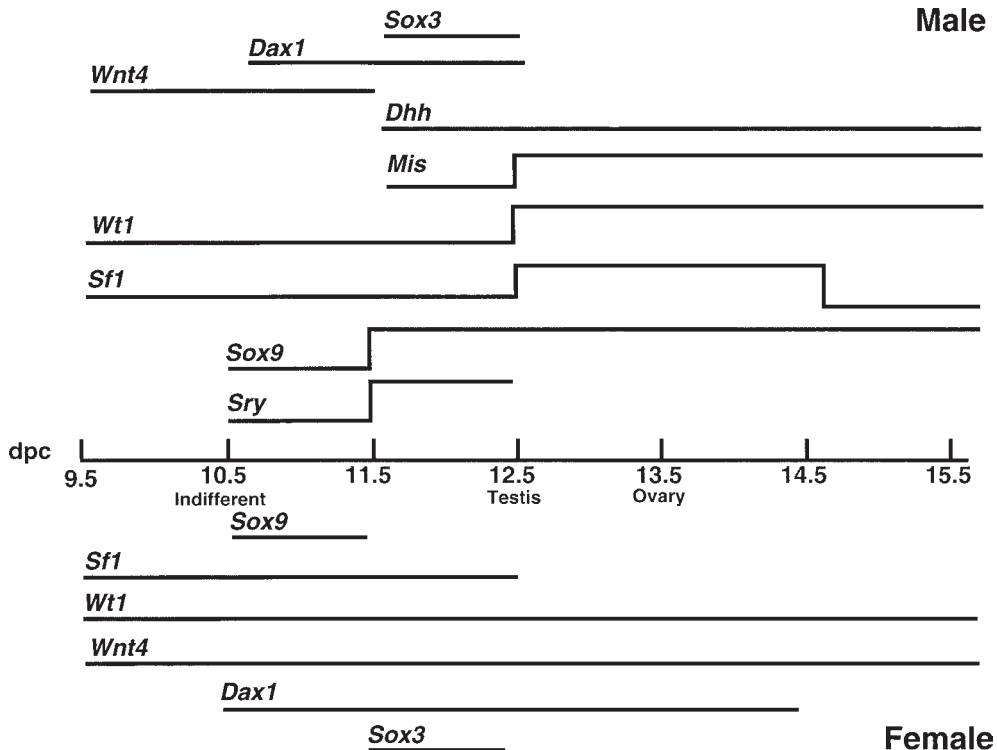


**Fig. 1.** Gonadal differentiation in the mouse. At 10.5 dpc in the mouse, the indifferent gonad contains four known cell types that are bipotential. The first stage of testicular differentiation occurs at 12.5 dpc, when the Sertoli cells differentiate from the supporting cell lineage to form cords that enclose the spermatogonia. Shortly after, Leydig cells differentiate from the steroidogenic cells and peritubular myoid cells arise from the connective cell lineage. Sertoli cells and Leydig cells produce MIS and testosterone, respectively, which are required for the development of the male internal reproductive structures. In the female at 13.0 dpc, the germ cells enter meiosis and are termed oocytes. Granulosa cells subsequently differentiate from the supporting cells, and thecal cells develop from the steroidogenic cells.

some—thus mapping the mutation to the testis-determining region of the Y. Consequently, this study provided the first case of XY sex reversal unequivocally caused by a mutation in *Tdy*.

EQUATING *SRY* WITH TDF

Major contributions to our understanding of sex determination and sexual differentiation in mammals have come from the study of mice and humans with primary sex reversal. The term “primary sex reversal” indicates XY individuals who develop as phenotypically female, despite the presence of a Y chromosome, and XX individuals who lack a Y chromosome but possess testicular tissue and consequently develop a male phenotype. Through analysis of the DNA of human patients with primary sex reversal, researchers have been able to progressively narrow down the portion of the Y chromo-



**Fig. 2.** Expression profiles of genes involved in sex determination and differentiation. Relative expression profiles of genes expressed in the supporting cell lineage and believed to be involved in sex determination and differentiation. Some genes, such as *Sf1* and *Dax1*, are also expressed in the steroidogenic cells; however, the expression profiles shown here are only representative of the supporting cell lineage and their descendants the Sertoli cells and granulosa cells.

some required to induce testicular differentiation in XX patients, which was missing in XY females (5,6). In “walking” along this 35-kb “minimal” testis-determining region, Sinclair and colleagues identified a gene they named *SRY*, which is now generally accepted to be TDF (6). In the mouse, *Sry* is absent from a strain in which XY individuals develop as females (7), and conversely, is present in a small fragment of the Y chromosome known to cause sex reversal in the  $XX^{Sxrb}$  mouse (7). *Sry* transcripts are detectable only within the male gonad, and during a very discrete period from 10.5–12.5 dpc, coincident with the onset of testicular differentiation at 12.5 dpc (Fig. 2) (8,9). With a few unique exceptions (10,11), *SRY* is conserved on the Y chromosome of all mammalian species examined (6,7,12). The *SRY* protein encodes an amino acid motif called the high-mobility group (HMG) box which confers its ability to bind to and bend DNA, and thus act as a transcription factor—a function in keeping with its role as a “switch” in the sex-determining pathway (7,8,13).

The most compelling evidence equating *SRY* with TDF came in the form of Randy, a transgenic mouse. When Koopman and colleagues microinjected into XX embryos a 14-kb fragment of the Y chromosome, containing only *Sry*, some of the resulting XX transgenics (including Randy) developed as phenotypic males complete with male

mating behavior (14). Thus, *Sry* is the only gene on the Y chromosome required for testis determination or, more specifically, for Sertoli-cell differentiation. In studies of  $XY^{Sry+} \leftrightarrow XY^{Sry-}$  chimaeras,  $XY^{Sry-}$  cells very rarely contribute to the Sertoli-cell population (reviewed in 15). Similarly, in the fetal gonads of  $XX^{Sxra}$  mice, in which the activity of the *Sxra* portion of the Y chromosome is influenced by the inactivation of the X chromosome, Sertoli cells display a preferentially active  $X^{Sxra}$  chromosome, while other testicular-cell types show a random inactivation of the X and  $X^{Sxra}$  chromosomes (16). Taken together these data reaffirm that *Sry* functions cell-autonomously within the pre-Sertoli cells to initiate Sertoli-cell differentiation.

### ***SRY* ALONE DOES NOT A TESTIS MAKE**

With the exception of *SRY* on the Y chromosome, all other genes involved in testicular differentiation are on the X chromosome or the autosomes—a fact demonstrated by the sex-reversed *Sry* XX transgenic mice. Intriguingly, only 30% of these mice sex reversed, and this phenomenon appears to be independent of transgene homozygosity or copy number (reviewed in 15). Over the course of breeding these mice, it became apparent that the background genotype of different strains of mice influences the propensity to sex-reverse (reviewed in 15). More specifically, this indicates an incompatibility between the timing and/or level of *Sry* expression and that of other genes on the X chromosome, or autosomes involved in either the testicular or ovarian differentiation pathway. A similar scenario occurs in the B6.Y<sup>Dom</sup> mouse, in which the Y chromosome from *Mus poschiavinus* (Y<sup>Pos</sup>) is placed onto an inbred *Mus musculus musculus* background (C57Bl/6).  $XY^{Pos}$  animals develop either as females with ovarian tissue or as hermaphrodites in which the gonads contain both ovarian and testicular tissue (17–19). Sex-reversal in these animals appears to be caused by a misregulation in the timing of *Sry* expression (20) and a functional incompatibility of *Sry*<sup>Pos</sup> with at least two autosomal alleles (19).

While there is no disputing the role of *SRY* in initiating testicular differentiation, it is a sobering consideration that only approx 20% of XY sex-reversals in humans can be attributed to mutations or deletions of *SRY* or its flanking sequences. Similarly, not all cases (80%) of XX males can be explained by the presence of *SRY* in the genome. Sex-reversal in XY females with an intact *SRY*—and in the remaining 20% of XX males lacking *SRY*—may be explained by a loss or gain of function, respectively, of autosomal or X-linked genes acting downstream, or upstream, of *SRY* in the testicular-differentiation pathway. In the next section, we discuss a number of genes which are thought to play a direct, or in some cases indirect, role in testicular differentiation: some of these factors appear to function upstream of *SRY*, and others to function downstream. We also introduce what little is known about the genes thought to be involved in ovarian differentiation. Of central importance is the contribution that transgenic studies in mice have made in defining the roles of each of the putative sex-determining and -differentiating genes discussed.

### **Wt1**

Heterozygous mutations of the Wilms' tumor-suppressor gene, *WT1*, are associated with Wilms' tumor, a childhood tumor of the kidney, and XY pseudohermaphroditism (reviewed in 21). The urogenital abnormalities in individuals with *WT1* mutations suggests a role for *WT1* in sex determination. *WT1* encodes a nuclear protein with domains

shared with transcription factors. In the mouse, *Wt1* expression is initially detected in the intermediate mesoderm that will give rise to the urogenital system, and later in the mesothelium and central nervous system (CNS) (22,23). In the developing gonad, *Wt1* is expressed in Sertoli cells (22). *Wt1* knockout mice are embryonic-lethal, and lack kidneys and gonads (24). It appears that the initiation of genital-ridge formation occurs, but quickly fails. Thus, *Wt1* appears to be essential for the initial formation of the gonads acting in males upstream of *Sry*. WT1 may also have functions later in male sexual differentiation by regulating genes such as *Mis* (25).

In the Denys-Drash syndrome, point mutations in the *WT1* locus that create missense, nonsense, or frameshift mutations result in more severe XY genital phenotypes in comparison to *WT1* null heterozygotes (26). Recently, the Denys-Drash syndrome has been modeled in mice by the introduction of a translation-stop codon at codon 396 of *Wt1* (27). However, only one heterozygous mouse carrying the T396 mutation was obtained from chimaeras. This heterozygote had small, aspermic testes. Unfortunately, this heterozygote also had sex-chromosome aneuploidy (XXY), complicating phenotype interpretations. Chimaeras generated with the T396 heterozygous ES cells also had genital abnormalities. This too was complicated by sex-chromosome chimaerism (XX $\leftrightarrow$ XY). The generation of mice carrying mutations that match those found in humans is a powerful approach for studying these mutations in vivo. However, it appears that these strategies may have to be modified to obtain interpretable information regarding sexual development.

### M33

A more recently identified candidate for a role in gonadal development and testicular differentiation is the mouse *M33* gene—a homolog of the *Drosophila Polycomb* gene (28). In *Drosophila*, *Polycomb* genes regulate the expression of homeotic genes that are required for segmental patterning in the embryo. Testicular differentiation in *M33* mutant mice is perturbed: gonadal phenotypes range from small ovaries to “indistinct” gonads, with some animals developing as hermaphrodites with one testis and one ovary (28). To date, *M33* mutant mice are the only example of recessive true hermaphroditism. Gonadal development is retarded in both XX and XY embryos at 11.5 dpc, when the expression of *Sry* in the male would normally reach its peak. At this stage, the gonads appear to consist of little more than a thickening of mesenchymal tissue, with an absence of coelomic epithelium. The coelomic epithelium is thought to give rise to Sertoli cells (29), and is thus likely to be the cell type in which *Sry* is expressed. Consequently, testicular differentiation in *M33* mutants may fail because of a paucity of coelomic epithelial cells, and subsequently, few or no Sertoli cells. Thus, the principal role of *M33* in the gonad appears to be in the process of gonadogenesis, with an indirect influence on testicular differentiation.

### Sox9

Heterozygous mutations in *SOX9* are responsible for the human skeletal-malformation syndrome, ampomelic dysplasia (CD) (30,31). Consistent with a role in testicular differentiation, approx 75% of XY patients with CD are sex-reversed (30–33). Gonadal phenotypes range from partial testicular differentiation to ovarian development with fewer than normal follicles to the most extreme of cases, where the gonads are reduced to streaks of fibrous tissue (34).



As with *SRY* and other members of the *SOX* (Sry-like HMG box) family of genes, *SOX9* encodes an HMG box. In addition, *SOX9* has in its carboxy terminus a transactivation domain (35). Patients with CD show a wide variety of mutations to *SOX9*, however in nearly all cases, the mutations result in a truncation of the transactivation domain (35). This suggests that *SOX9* is required for the transactivation of downstream genes involved in skeletal and testicular development. The disparate nature of the mutations, and the observation that all patients are heterozygous for the mutation, suggest that CD and its associated sex reversal are caused by a haploinsufficiency for *SOX9* rather than a dominant-negative effect.

In the mouse, low levels of *Sox9* expression are seen in both male and female urogenital ridges from 10.5 dpc, around the time the development of the indifferent gonad (Fig. 2) (36,37). A sexually dimorphic pattern of expression begins at 11.5 dpc, when levels in the male gonad increase, coincident with the peak in *Sry* expression, while in the female, expression of *Sox9* is turned off. At 12.5 dpc, *Sox9* transcripts in the testis are localized to the Sertoli cells. The *SOX9* protein is conserved among the vertebrate groups with nonmammalian sequences thus far identified for chicken, turtle, and alligator (38–40). Expression of *SOX9* in these species is similarly correlated with testicular differentiation. However, in contrast to the pattern seen in mammals, *SOX9* expression in chickens and alligators occurs later in Sertoli-cell differentiation, and after the production of MIS (38,40). These data suggest that while *SOX9* appears to play a role in instigating Sertoli-cell differentiation in mammals, in reptiles and birds it may function later in development—perhaps to maintain, rather than determine, Sertoli-cell fate.

We are addressing the role of *Sox9* in Sertoli-cell differentiation in the mouse, using both loss-of-function and gain-of-function approaches. Our loss-of-function study uses gene targeting in ES cells to generate mice with only one functional copy of *Sox9*. More specifically, we have deleted approx 450 bp of *Sox9* including the translation start site and the majority of the HMG box, thus generating a null allele (41). Testicular development in mice heterozygous for the null allele is normal, while skeletal development is disrupted. This situation stands in contrast to the human condition, where the vast majority of XY patients with mutations of *SOX9* show both sex-reversal and skeletal malformations.

In a further attempt to understand the role of *Sox9* in Sertoli-cell differentiation, we have generated ES cells that are homozygous for the *Sox9* mutation. In chimaeras, *Sox9*-null ES cells give rise to Sertoli cells, indicating that *Sox9* is not required cell-autonomously for Sertoli-cell differentiation.

We have also generated fetuses that are homozygous for the *Sox9* mutation by injecting *Sox9*-null ES cells into blastocysts that are derived from tetraploid cells. In this instance, the embryo will be entirely ES-cell-derived, with tetraploid cells contributing only to the extraembryonic tissues. *Sox9*-null fetuses are grossly abnormal, and die shortly after 10.5 dpc. However, the gonad at this time appears to be normal, indicating that early expression of *Sox9* is not required for the development of the indifferent gonad.

More direct data implicating *Sox9* in Sertoli-cell differentiation has resulted from our gain-of-function study. In these mice, a tyrosinase minigene has fortuitously inserted within 1 centimorgan of *Sox9* (42). All XX mice carrying the transgene (XXtg) develop as phenotypically normal, although sterile, males. Significantly, all of these mice have a normal XX karyotype, and are devoid of known Y chromosome genes, including *Sry*. Gonadal development in the XXtg males follows the typical male pathway from the

outset, with Sertoli cells and early seminiferous cords visible at 12.5 dpc. The differentiating Sertoli cells express *Sox9* and MIS. By 15.5 dpc, the XXtg testis contains well-organized seminiferous cords enclosed by peritubular myoid cells, interstitial tissue, and a tunica albuginea. In wild-type animals at 11.5 dpc, Sertoli cells have not yet differentiated; this state of morphological development corresponds with an upregulation in the male, and a downregulation in the female, of *Sox9* expression. In the XXtg gonad at 11.5 dpc, *Sox9* expression persists at high levels. These data indicate that the insertion of the transgene has potentially disrupted a repressor element upstream of *Sox9*, which would normally allow for the expression of *Sox9* to be downregulated in the female. Continued expression of *Sox9* in the XXtg gonad initiates testicular differentiation in the absence of *Sry*, implicating *Sox9* as a gene involved in the early stages of Sertoli-cell differentiation, perhaps immediately downstream of *Sry*.

If we extrapolate further, we can propose a model with *Sox9* as the critical testis-differentiating gene, and the function of *Sry* is to ensure that *Sox9* is upregulated in the XY gonad—perhaps by repressing a negative regulator of *Sox9* expression. In the XX gonad, this repression of *Sox9* expression would persist, preventing testicular differentiation in the presumptive ovary. In our XXtg mice, the insertion of the transgene may have disrupted the binding site for this repressor of *Sox9*, thereby permitting its continued expression. The role of *Sry* as a repressor of a repressor of testicular differentiation was originally proposed by McElreavey and colleagues in 1993, as an alternative to the more conservative view that *Sry* acts as a dominant testis inducer (15). The reader will find that as we progress further into this chapter, the concept of *Sry* as an antagonist of an “anti-testis” factor becomes an increasingly attractive proposal.

### ***Sf1 and Dax1: Multifunctional Regulators of Gonadal Development and Function***

In addition to *Wt1* and *M33*, a strong candidate for a role in early gonadogenesis is the *Ftz-F1* gene, which encodes the orphan nuclear-receptor steroidogenic factor 1 (SF1). In the fetus, SF1 has multiple roles in early gonadal, adrenal, and brain development, and throughout life is involved in the regulation of steroidogenesis in multiple tissues (*see* 43 and Chapter 8 for review). Mice homozygous for a null mutation in *Sf1* lack adrenals, show gonadal degeneration after 11.5 dpc, have impaired gonadotrophic function, and altered structural characteristics of the ventromedial hypothalamus (44–47). Taken together, these studies demonstrate an essential role for *Sf1* at multiple levels of the hypothalamic-pituitary-gonadal axis.

At 9.0 dpc in the mouse, *Sf1* transcripts are present in the urogenital ridge, and expression continues in the indifferent gonad of both sexes (Fig. 2) (48). A sexually dimorphic pattern of expression becomes apparent at 12.5 dpc, when *Sf1* transcription increases in the developing testis and is downregulated in the prospective ovary. At 14.5 dpc, *Sf1* transcripts in the testis are restricted to the Leydig cells. In the female, expression of *Sf1* is reinstated predominantly in the thecal cells of the postnatal ovary. The diminished expression of *Sf1* in the ovary at 12.5 dpc is coincident with the onset of testicular differentiation in the male, and suggests that SF1 may regulate the expression of testis-differentiating genes that are disruptive to ovarian development.

The expression profiles of *Sf1* overlap considerably—both temporally and spatially—with that of another orphan nuclear receptor, *Dax1*. Mutations in *DAX1* are responsible for the human disorder X-linked adrenal hypoplasia congenita (AHC), which is charac-



terized by an absence of the adrenal cortex and hypogonadotropic hypogonadism (49). In the mouse, *Dax1* is first expressed in the somatic cells of the indifferent gonad of both sexes at approx 10.5 dpc (Fig. 2) (50,51). At 12.5 dpc, levels of *Dax1* expression in the developing testis decrease significantly to levels that are barely detectable. In the female gonad, *Dax1* continues to be expressed until approx 14.5 dpc. In the adult testis and ovary, *Dax1* transcripts are restricted to the steroidogenic Leydig and thecal cells, respectively. Similar relative expression profiles for *DAX1* and *SF1* are conserved in the pig (52). The inverse expression profiles of *Dax1/DAX1* and *Sf1/SF1* during testicular differentiation, and their co-expression in the steroidogenic cells of the testis and ovary, imply that these genes interact during gonadal development and endocrine function.

SF1 and DAX1 have been shown to act antagonistically in the regulation of *Mis* expression in the developing testis. Previous studies have demonstrated that SF1 is required for the expression of *Mis* in vitro and in vivo (53,54). More recently, Nachtigal and colleagues (25) have shown that DAX1 antagonizes SF1-mediated transactivation of *Mis*. Specifically, SF1 and the transcription factor WT1 are thought to associate and thereby synergistically promote *Mis* expression. DAX1, however, antagonizes this synergy, probably by interacting directly with SF1. Further, in vitro studies from several laboratories have indicated that DAX1 can inhibit the transcriptional activation by SF1 of genes involved in steroidogenesis (55–57). It is suggested that DAX1 is able to bind to DNA hairpin-loop structures (56). This DNA-binding ability is shared by SRY. Thus, a likely scenario might depict SRY and DAX1 influencing the ability of each other to either activate or repress SF1-mediated transcription of genes involved in steroidogenesis and testicular differentiation.

In addition to its antagonistic effects on SF1 transcriptional activity, *DAX1* has also been implicated as an ovary-determining gene, or “anti-testis” gene. *DAX1* maps to a 160-kb region of the X chromosome which, when duplicated, causes XY-dosage-sensitive sex reversal (DSS) (49,58,59). In vivo confirmation that *DAX1* is indeed the gene responsible for DSS was obtained by generating transgenic mice expressing a *Dax1* cDNA under the regulation of a fragment of DNA taken upstream from the *Dax1* start of transcription: this transgene was designated Dax:Dax (60). In one transgenic line, the levels of *Dax1* expression were five times that of normal levels, with a corresponding increase in protein levels. Surprisingly, all XY transgenics from this line developed testes, although development was initially retarded. However, when transgenic females were bred to males carrying a weak *Sry* allele ( $Y^{Pos}$ ), 3 of 14 liveborns developed as hermaphrodites and eight developed as males, and at least four of these probably possessed ovotestes earlier in development. In fact, when fetal gonads were examined, 8 out of 11  $XY^{Pos}$  transgenics had ovotestes. Similar results were obtained when the *Dax1* promoter was used to express *Sry* (Dax:Sry transgene) in XX animals carrying Dax:Dax.

Paradoxically, XY mice homozygous for the *Dax1* transgene, and expressing levels of DAX1 that are approx 11 times higher than endogenous levels, still develop normal testes. Yet in humans, a double dose of DAX1 is sufficient to induce a sex-reversal. Testicular development is only disrupted in those transgenic mice that possess a weak *Sry* allele ( $Y^{Pos}$  or Dax:Sry). The authors give a plausible account that takes into consideration two important observations. Firstly, testis development in both the  $Y^{Pos}$  and Dax:Sry mice lacking the Dax:Dax transgene is delayed. This becomes significant when one also takes into account that transcription of *Sry* in wild-type animals appears to pass along the gonad in such a way that each cell expresses high levels of *Sry* for only a brief

period. Thus, most cells will have experienced the peak in *Sry* expression, and become committed to the Sertoli-cell fate, before experiencing high levels of *Dax1* expression. Consequently, *Sry* is able to outcompete *Dax1* for the activation of testis-differentiating genes. Secondly, in the case of Dax:Dax transgenics with the  $Y^{Pos}$  *Sry* allele, where levels of transcription are lower than normal, or XX Dax:Sry animals, where the onset of *Sry* expression appears to be delayed, *Dax1* expression increases before that of *Sry*, allowing *Dax1* to maintain its inhibition of testis-differentiating genes. Although the experiments are not optimal, these data suggest that DAX1 and SRY act antagonistically toward each other, competing to control the activation of genes in the testicular-differentiation pathway.

In apparent contradiction to the above hypotheses, mice deficient for *Dax1* develop normal ovaries and internal reproductive structures (61). The only abnormality in females appears to be that some follicles contain more than one oocyte. These data do not support a role for *Dax1* as an ovary-determining gene. On the other hand, it is still plausible that DAX1 can act as an “anti-testis” factor by interfering with the expression of testis-differentiating genes, such as *Sox9*. Very low levels of *Sox9* expression are seen in both male and female urogenital ridges at 10.5 dpc, coincident with the expression of *Sf1*. It is possible that SF1 directs this very low level of *Sox9* expression. At 10.5 dpc, *Dax1* is expressed in male and female indifferent gonads, corresponding with the onset of *Sry* expression in the male. Critically, at 11.5 dpc, levels of *Sry* expression peak and *Sox9* levels are substantially elevated. In the female at this time, expression of *Sox9* is extinguished. If we assume that SF1 is responsible for maintaining low levels of *Sox9* expression, DAX1 may heterodimerize with SF1 and counter SF1-mediated transcriptional activation of *Sox9*. SRY may antagonize this effect of DAX1, by competing for the same binding site or altering the conformation of the DNA in such a way that DAX1 cannot bind or dimerize with SF1. Most importantly it may directly, or indirectly, cause the substantial increase in *Sox9* expression seen in the male at 11.5 dpc. This boost in *Sox9* expression is essential for testicular differentiation to occur. In the loss-of-function females lacking *Dax1*, expression of *Sox9* may continue at lower levels, but without the boost in *Sox9* expression seen in the male, the gonad continues along the ovarian-differentiation pathway. The expression of *Sox9* in the gonads of *Dax1*-deficient females is unknown.

Another possibility is that another gene, or genes, is also involved in the repression of *Sox9* expression in the female gonad, and that in the absence of *Dax1*, this other factor is sufficient to keep *Sox9* levels below the threshold for testicular differentiation. A possible candidate for this role is the *Sry*-related gene *Sox3*.

### ***Sox3: Ancestor of Sry and a Putative Ovary Determinant***

Of all the *SOX* genes, *SOX3* is most closely related to *SRY*, sharing 82% similarity in the HMG box (62,63). Both *SOX3* and *SRY* are encoded by one exon, and share homology outside of the HMG box—an intriguing finding, because no such homology exists between species for *SRY*. It has been proposed that, in mammals, *SRY* evolved from *SOX3* during the evolution of the X and Y chromosomes (64,65). *SOX3* has been highly conserved throughout vertebrate evolution, and is X-linked in both eutherian and marsupial mammals (62–64,66).

The predominant site of *Sox3* expression in the mouse is in the developing central nervous system (63). However, at 11.5 dpc, *Sox3* expression is observed in both male and female genital ridges (Fig. 2). Transcripts are localized to the somatic cells of the gonad

and the expression level in the female appears to be twice that of the male, reaching levels equivalent to or greater than *Sry*. By 12.5 dpc, transcripts are no longer detectable in either male or female gonads (A. Hacker unpublished data, cited in 63).

In vitro data indicate that SOX3 and SRY bind to the same DNA target sequence; however, SOX3 binds with a much lower affinity than SRY (63). Thus, a likely scenario may be one in which SOX3 and SRY compete for the same target sequence involved in the regulation of ovarian- or testicular-differentiation genes. More specifically, Jennifer Graves (65) has proposed that SOX3 and SRY compete to regulate the expression of *SOX9*. In her model, SOX3 was once part of a dosage-regulated system of sex determination that involved the differential regulation of *SOX9*. With the evolution of *SRY* from *SOX3*, a more robust mechanism of sexual differentiation came into play. Thus, in the female, SOX3 would repress the expression of *SOX9* and consequently, other testis-differentiating genes. In the male, SRY would outcompete SOX3 for its binding site in the *SOX9* promoter region, and testicular differentiation would ensue. To date, nothing is known about the presence or absence of such a binding site in the *SOX9* promoter. XY human individuals deleted for *SOX3* develop small, but essentially normal, testes (62); XY individuals with a duplication of *SOX3*, or XX individuals deleted for *SOX3* have not been identified. However, loss-of-function and gain-of-function studies in mice will contribute substantially to defining the requirement of *Sox3* for ovarian differentiation.

### Mis

MIS is one of the key hormones required in male development (*see* 67 and Chapter 3 for review). MIS is produced by the Sertoli cells of the fetal testis, with the highest levels observed during the period in which the Müllerian duct regresses (68,69). Expression of MIS continues after birth at reduced levels, and then declines sharply at puberty (69–72). In the mouse, *Mis* transcripts are first detected in the differentiating Sertoli cells at 11.5 dpc, 1½ d before the Müllerian ducts begin to regress (Fig. 2) (69).

Initially, transgenic mice were used to explore the potential roles of MIS in vivo (73). The mouse metallothionein promoter (MT) was used to direct widespread expression of human MIS (hMIS). Female MT-hMIS transgenic mice were born without a uterus or oviducts as expected. In addition, the ovaries became depleted of germ cells soon after birth, and eventually degenerated. These findings further confirmed the role of MIS in Müllerian-duct regression, and demonstrated that high levels of MIS were directly, or indirectly, toxic to female germ cells. Although most male MT-hMIS transgenic mice were overtly normal and fertile, some males from the highest expressing lines did not virilize and had undescended testes. Lyet et al. (74) determined that the high levels of hMIS caused a reduction in circulating testosterone. Presumably, those nonvirilized males from the highest expressing lines had severely reduced testosterone levels. More recently, Racine and colleagues (75) have shown that the overexpression of hMIS in male MT-hMIS transgenics blocks the differentiation of Leydig-cell precursors and decreases expression levels of the cytochrome p450 17 $\alpha$ -hydroxylase gene, which is required for steroid synthesis. MIS appears to exert its effects on Leydig-cell differentiation and steroidogenesis directly via its receptor, which is now known to be expressed by Leydig cells in addition to Sertoli cells (75).

*Mis* knockout mice have also been generated (76). Although *Mis* is specifically expressed in postnatal granulosa cells of the ovary, *Mis*-mutant females are normal and fertile. However, it is possible that more subtle alterations in ovarian function are present.

*Mis*-mutant males developed as male pseudohermaphrodites, possessing testes and male internal organs as well as a uterus and oviducts. The majority of the *Mis*-mutant males proved to be infertile, not because of a germ-cell deficiency, but because of the physical abnormalities caused by the codevelopment of both male and female internal reproductive organs. In addition, a proportion of the older *Mis*-mutant males had Leydig-cell hyperplasia, indicating that *Mis* also influenced Leydig-cell proliferation. The finding that MIS receptors are expressed on Leydig cells suggests that this might be a direct interaction between Sertoli and Leydig cells (75). By generating *Mis* mutant males that also carried the testicular feminization (*Tfm*) mutation, XY animals were produced that could not respond to the two primary male hormones: testosterone and MIS. The resulting animals overtly appeared as females, with a uterus and oviducts, but no Wolffian-duct derivatives. Testes were intra-abdominal at the position where ovaries would normally be located. These studies re-emphasized the hormonal control of sexual differentiation in mammals.

TGF $\beta$  signaling is mediated by transmembrane receptors with serine/threonine kinase activity (77). Type I and type II receptors generate complexes upon ligand binding, and transduce their signals through Smad proteins to the nucleus. The type I receptor for MIS is unknown; however, the type II receptor for MIS has been isolated in the mouse (78), rat (79,80), rabbit (81), human (82), and tammar wallaby (D. Whitworth, unpublished data). We have generated MIS type II receptor knockout mice (83). Interestingly, these mice are a phenocopy of the MIS ligand knockout mice, suggesting that the MIS signaling pathway involves a one ligand-one type II receptor pathway. The specificity of MIS signaling was established by generating female MT-hMIS mice that were also deficient for the MIS type II receptor (84). In contrast to MT-hMIS females that lack a uterus, oviducts, and ovaries, the MT-hMIS/MIS type II receptor-mutant females were normal and fertile.

In the mouse, *Mis* expression is detected around 11.5 dpc, specifically in preSertoli cells and later in Sertoli cells of the fetal and postnatal testes (9). The timing and cell specificity of *Mis* expression suggested that *Sry* may directly or indirectly regulate *Mis* transcription. However, tissue-culture and transgenic mouse experiments have indicated that steroidogenic factor 1 (SF1) is required to activate *Mis* transcription (53,54). In contrast, targeted mutagenesis of the SF1 binding site in the endogenous mouse *Mis* locus does not block *Mis* transcription, but rather reduces *Mis* transcription levels (85). This reduction is not sufficient to cause a persistence of Müllerian-duct-derived tissues. However, when a conserved SOX9 binding site is similarly mutated, no *Mis* transcription occurs and the mutant males develop as pseudohermaphrodites, similar to *Mis* mutant males. These manipulations of the endogenous promoter have revealed the distinct activities of these two transcription-factor binding sites in regulating *Mis* expression and have helped to piece together this important genetic pathway of male sexual differentiation.

### **Tfm**

Mice carrying the X-linked *Tfm* mutation have female genitalia and lack Wolffian duct-derived structures, despite possessing testes and an XY karyotype (86). *Tfm* is a naturally occurring mutation to the androgen receptor (AR) that results in complete androgen insensitivity (reviewed in 87 and 88). In the absence of a functional AR, neither testosterone nor its more potent metabolite 5 $\alpha$ -dihydrotestosterone can exert their effects on the Wolffian duct and the external genitalia, respectively. At the molecular level, *Tfm* is a single-base deletion in the N-terminal region of the AR gene. This leads to a premature termination of translation, which in turn gives rise to a truncated, unstable mRNA transcript (89–92).

The external genitalia of *Tfm* male mice resembles that of wild-type females, although the vagina is shorter than normal and often blind-ending (86). Testes of adults are smaller, and fail to descend beyond the internal inguinal ring into the scrotum (reviewed in 88). Spermatogenesis proceeds in the testes of *Tfm* mutants; however, most germ-cells fail to progress beyond the spermatocyte stage (86). Levels of the pituitary gonadotrophins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are elevated in *Tfm* males because of the loss of the AR-mediated negative feedback loop, which would normally regulate their production (reviewed in 88). Leydig cells appear to be normal in number, but are hypertrophied (86). In addition, the production of androgens is severely reduced, probably a result of the loss of the enzyme 17 $\alpha$ -hydroxylase (93).

By generating XX mice that are sex-reversed (XX<sup>Sxr</sup>) and carrying the *Tfm* mutation, Ohno and colleagues were able to study the interactions during male development between cells that are wild-type for the AR, and those that are *Tfm*-mutated (reviewed in 87). Because of X-inactivation, sex reversed mice that are heterozygous for *Tfm* are mosaics of cells which are sensitive to androgens (X<sup>WT</sup>) and those that are insensitive (X<sup>Tfm</sup>). The conclusion from these studies was that effects of testosterone could be mediated by local growth factors from wild-type cells to *Tfm* cells (reviewed in 87). While cellular differentiation was seen to occur only in wild-type cells, embryonic induction, the morphogenesis of male reproductive structures, and the postnatal maintenance of these structures were all found to be mediated effects. The *Tfm* mouse provides an excellent model in which to study the breadth and mechanics of androgen function.

### Wnt4

WNT4 belongs to the WNT family of secreted glycoproteins, with members that function during development as signaling molecules in a diverse range of cell types (94). In the mouse, *Wnt4* expression is first detected at 9.5 dpc in the mesenchyme and coelomic epithelium of the mesonephros (95). Shortly afterwards, expression of *Wnt4* extends into the mesenchyme of the indifferent gonad. Transcription of *Wnt4* in the gonads becomes sexually dimorphic at 11.5 dpc, when it is downregulated in the male gonad but maintained in the somatic cells of the female gonad.

In the female, *Wnt4* appears to be required for suppressing the differentiation of Leydig cells from the steroidogenic cell lineage. Ovaries collected from *Wnt4* homozygous mutant females at 14.5 dpc express the steroidogenic enzymes 3 $\beta$ -HSD and P450c17, both of which are involved in the synthesis of testosterone by Leydig cells and their precursors, and are also expressed by steroidogenic cells of the adult ovary. These ovaries also express type III 17 $\beta$ -HSD which, unlike 3 $\beta$ -HSD and P450c17, is a testis-specific enzyme. The Wolffian ducts of *Wnt4* mutant females persist and become coiled at their most cranial end, so that they resemble the epididymides of the male. This further suggests that the ovaries of these animals produce testosterone during fetal development. Lastly, *Wnt4* also has a role in the early formation of the Müllerian duct in both sexes. At 12.5 dpc, male and female *Wnt4* mutants lack an identifiable Müllerian duct and do not express the Müllerian duct markers *Pax8* and *Wnt7a*.

### Wnt7a

Another member of the WNT family, WNT7a, also appears to be involved in Müllerian-duct development. In the fetus, *Wnt7a* is expressed in the epithelium of the Müllerian duct in both males and females from 12.5 dpc to 14.5 dpc (96). In the



male, expression of *Wnt7a* diminishes as the Müllerian duct regresses, while in the female expression persists into the adult where it is localized to the epithelial cells of the Müllerian-duct-derived oviducts and uterus (96,97). Development of the Müllerian duct in *Wnt7a* homozygous mutant females is abnormal (96). At birth, these mutant females lack coiled oviducts, and the wall of the uterus is thinner than that of wild-type littermates. Similarly, in adult females, the oviducts are still abnormal, the uterus is nearly devoid of uterine glands, and the stromal layer is thinner.

Male homozygous mutants have persistent Müllerian ducts resembling those seen in the MIS and MIS type II receptor mutants. Critically, *Wnt7a* mutant males (and females) fail to express the MIS type II receptor in the mesenchyme surrounding the Müllerian duct. This suggests that expression of *Wnt7a* in the epithelial cells of the Müllerian duct is required for regulating the expression of the MIS type II receptor in the periductal mesenchyme which, in turn, induces regression of the Müllerian duct epithelium.

### Dhh

Hedgehogs are important signaling molecules that regulate diverse developmental processes (98). Vertebrate hedgehog genes include *Sonic (Shh)*, *Indian (Ihh)*, and *Desert (Dhh)*. It is *Desert hedgehog* that is most relevant to mammalian sex determination and differentiation. At the moment, *Dhh* has only been reported in mice, however *DHH* has also been isolated from a marsupial mammal, the tammar wallaby (C.-A. Mao, unpublished data). In the mouse, *Dhh* expression is initially detected in the fetal testis at 11.5 dpc (Fig. 2). Later, in the adult testis, *Dhh* is found specifically in Sertoli cells. *Dhh* is also detected in other tissues of the developing mouse embryo, including Schwann cells, the endothelium of the vasculature, and the endocardium (99). The highly restricted expression pattern of *Dhh* in the somatic cells of the male gonad suggests that *Dhh* may be an important regulator of the male phenotype.

*Dhh* mutant mice have been generated by gene targeting in ES cells (100). Male *Dhh* mutants were found to be viable yet sterile because of a disruption to spermatogenesis. Interestingly, the expression of *Patched (Ptc)*, which encodes the receptor for hedgehogs, is normally detected in the Leydig cells. In the *Dhh* mutants, *Ptc* was not detected. These findings suggest that DHH produced by Sertoli cells interacts with Leydig cells to support spermatogenesis. Therefore, the defects in spermatogenesis may be caused by alterations in androgens that are required for male germ-cell development. This would have to be an androgen deficiency that only affects spermatogenesis because the *Dhh* mutant males (at least on the genetic backgrounds analyzed) were normally virilized. These results also indicate that *Dhh* is not involved in sex determination, but rather sex differentiation and spermatogenesis. The initiation of *Dhh* expression in the somatic cells of the male fetal gonad occurs soon after the initiation of *Sry* expression, suggesting that SRY regulates *Dhh*, either directly or indirectly. Therefore, it will be interesting to determine the *cis*- and *trans*-acting factors required to direct Sertoli-cell-specific transcription of *Dhh*.

### TRANSGENIC MARSUPIALS: A NEW FRONTIER

Sexual development in marsupials is fundamentally similar to that described for eutherian mammals such as the mouse and human. However, there are some significant differences in the morphology of the reproductive structures and in the timing of events. It is our goal to exploit these differences in order to gain a more detailed insight into mammalian sexual development from an alternative perspective.



In marsupials, as in eutherians, the Y chromosome is testis-determining (101,102), presumably under the direction of the marsupial homolog of *SRY* (12). Testicular differentiation precedes ovarian differentiation—as is the case in eutherians—and the development of the internal reproductive structures is similarly under the influence of MIS and steroids. However, in marsupials, these events occur after birth. In addition, there are some sexually dimorphic structures in the marsupial that appear to be under direct genetic—rather than secondary gonadal—hormonal control. In at least four species of marsupials, including the South American grey opossum (*Monodelphis domestica*), North American Virginia opossum (*Didelphis virginiana*), and the Australian brushtail possum (*Trichosurus vulpecula*) and tammar wallaby (*Macropus eugenii*), clearly defined scrotal and mammary anlagen are visible several days before gonadal sex differentiation (103–106). Many species of marsupials develop a pouch in the female. In these species, pouch development similarly appears to be independent of gonadal sex.

Descriptions of spontaneously occurring intersexes provide further evidence for the direct genetic control of these sexual dimorphisms in marsupials. Several studies (102,107–109) together describe five intersexes from five different marsupial species with either an XXY, XXY/XX/XY, or aberrant XY karyotype. Each individual possessed functionally normal testes, as evidenced by the presence of a normal male reproductive tract, and yet lacked a scrotum, while possessing a pouch and mammary glands. Taken together, these observations provide compelling evidence that the sexually dimorphic development of the scrotum, pouch, and mammary glands is under direct genetic control, rather than hormonally mediated, as is the case in eutherians. In short, development of the scrotum occurs when only one X chromosome is present (XY, XO), while in the presence of two X chromosomes (XX, XXY) a pouch and mammary glands form (107,110,111).

Using the South American grey opossum, it is possible to rigorously test this hypothesis by injecting *M. domestica* eggs with an *M. domestica* *SRY* transgene. XX transgenic animals would be expected to have testes, but the key issue will be whether or not they develop a scrotum. If a scrotum does form, this would suggest that scrotal development can be induced by the presence of a testis, thus refuting the current theory that scrotal development in marsupials is independent of gonadal sex.

Research interest in marsupials is not only restricted to the field of sexual differentiation. Marsupials are also unique in comparison to eutherian mammals in their pre-implantation development, placentation, the development of their reproductive organs, and X chromosome imprinting (reviewed in 112–114). By establishing transgenic techniques in marsupials, we will generate an exceptional resource for comparative studies of the genetic control of mammalian pre- and postimplantation development.

## CONCLUSION

The progression from indifferent gonad to testis or ovary is dependent upon a suite of cellular migration, differentiation, and endocrine events. The challenge in understanding how the testis and ovary come into being is in piecing together and integrating what we know at the morphological and molecular levels. From a morphological perspective, it is becoming clearer that differences between XX and XY indifferent gonads in the proliferation of coelomic epithelial cells, the migration of cells into the gonad from the mesonephros, and the cross-communication between germ cells and somatic cells, are all important factors in the development of the testis and ovary. As we learn more about

Table 1

## Gene Functions and References to Studies of Transgenic Mice and Natural Mutants

<i>Gene</i>	<i>Function</i>	<i>Transgenic studies</i>
<i>Sry</i>	Sertoli cell fate	(4,14)
<i>Wt1</i>	Gonadogenesis/Regulation of <i>Mis</i> expression	(24,27)
<i>M33</i>	Gonadogenesis	(28)
<i>Sox9</i>	Sertoli-cell fate/Regulation of <i>Mis</i> expression	(42)
<i>Mis</i>	Müllerian-duct regression	(54,73–75,84,85)
	Leydig-cell differentiation	
	Steroidogenesis	
<i>MIS Type II Receptor</i>	MIS signaling	(83,84)
<i>AR (Tfm)</i>	Androgen signaling	(86,89,90–92)
<i>Wnt4</i>	Müllerian-duct formation	(95)
	Suppression of Leydig-cell differentiation in ovary	
<i>Wnt7a</i>	Müllerian-duct regression and differentiation	(96)
<i>Dhh</i>	Spermatogenesis	(100)
<i>Sfl</i>	Gonadogenesis	(44–47)
	Sertoli-cell and Leydig-cell endocrine function	
<i>Dax1</i>	Leydig-cell endocrine function	
	Granulosa-cell fate and endocrine function	(60,61)

the discrete morphological changes that comprise the grand process of sexual differentiation, it becomes an easier prospect to isolate and place into order candidate genes and the functions of their protein products.

At the molecular level, SRY is undoubtedly testis-determining, but we still don't know whether it acts as a dominant testis-inducer or as an inhibitor of ovarian development, and so, by default, is an indirect testis-determinant. SF1 and WT1 appear to each play multiple roles in both the early stages of gonadogenesis, and later in the regulation of testicular and ovarian endocrine function (Table 1). SOX9 appears to be essential not only in determining Sertoli-cell fate, but also in regulating their production of MIS. Importantly, *SOX9* expression in the embryo is not restricted to the testis and so the testis-specific expression of MIS must also require the collaboration of other factors. In addition to its long-known role in inducing the regression of the Müllerian ducts, MIS has more recently been credited with additional functions in regulating the proliferation of Leydig cells and their production of testosterone. The function of MIS in the postnatal ovary is speculative at best. Indeed, our knowledge of the molecular events required for granulosa-cell differentiation is sorely lacking. DAX1 may be important to granulosa cell fate by acting as an antagonist to SRY, while postulated roles for SOX3 remain to be supported by loss- or gain-of-function analyses in humans and mice. Thus, the picture of the molecular aspects of sexual differentiation that begins to emerge is one of an exceedingly complex meshing of factors—many with multiple functions—rather than a straightforward linear hierarchy of interactions.

The difficulty in piecing together a model of molecular events to explain the morphological changes observed in the differentiating gonad is that we know relatively few of the genes which must be involved in this process. As new candidate genes are identified

and put to the test by gain- and loss-of-function studies in mice, we come closer to being able to integrate the molecular with the morphological. Comparative studies between different eutherian species and marsupials will allow us to test the generality of models extrapolated from the mouse.

## ACKNOWLEDGMENTS

We are grateful to Soazik Jamin and Alex Arango for their thoughtful suggestions during the preparation of this manuscript. Work from Richard R. Behringer's laboratory discussed herein was supported by a grant from the NIH (HD30284).

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Transgenics in Endocrinology

Matzuk, M.; Brown, C.W.; Kumar, T.R. (Eds.)

2001, X, 486 p., Hardcover

ISBN: 978-0-89603-764-9

A product of Humana Press