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Sex Determination and Gonadal Development in Mammals

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Wilhelm D, Palmer S, Koopman P. Sex Determination and Gonadal Development in Mammals. *Physiol Rev* 87: 1–28, 2007; doi:10.1152/physrev.00009.2006.—Arguably the most defining moment in our lives is fertilization, the point at which we inherit either an X or a Y chromosome from our father. The profoundly different journeys of male and female life are thus decided by a genetic coin toss. These differences begin to unfold during fetal development, when the Y-chromosomal *Sry* ("sex-determining region Y") gene is activated in males and acts as a switch that diverts the fate of the undifferentiated gonadal primordia, the genital ridges, towards testis development. This sex-determining event sets in train a cascade of morphological changes, gene regulation, and molecular interactions that directs the differentiation of male characteristics. If this does not occur, alternative molecular cascades and cellular events drive the genital ridges toward ovary development. Once testis or ovary differentiation has occurred, our sexual fate is further sealed through the action of sex-specific gonadal hormones. We review here the molecular and cellular events (differentiation, migration, proliferation, and communication) that distinguish testis and ovary during fetal development, and the changes in gene regulation that underpin these two alternate pathways. The growing body of knowledge relating to testis development, and the beginnings of a picture of ovary development, together illustrate the complex mechanisms by which these organ systems develop, inform the etiology, diagnosis, and management of disorders of sexual development, and help define what it is to be male or female.

I. INTRODUCTION

When a child is born, most often the first question asked is: Boy or girl? From that moment forward, our sex, whether we are male or female, influences almost every aspect of who we are and how we live. But how deep are the differences between males and females, and how do these differences come about? These questions seem at first glance rather simple, but in fact they are stunningly complex.

The essential purpose of sexual differentiation, the development of any male- or female-specific physical or behavioral characteristic, is to equip organisms with the necessary anatomy and physiology to allow sexual reproduction to occur. As far as the genetics of sexual development is concerned, arguably the most significant events unfold within the interior world of the fetal gonads. Although in mammals the sexual fate of the organism is cast at fertilization, this fate is revealed only during fetal development, when the gonads begin to differentiate as

ovaries or testes after a considerable period of sexual ambiguity. All secondary sexual dimorphisms are thought to follow from the differentiation of the gonads and their acquisition of endocrine function.

In 1947, Alfred Jost demonstrated that if XX and XY rabbit fetuses were castrated in utero before sexual differentiation, they went on to develop ducts and external genitalia of the female pattern. Therefore, development of femaleness represents the "default" state and is independent of gonadal hormones. This essential piece of work demonstrated that the question of how sexual differentiation is achieved can be posed more simply by asking what determines a testis or an ovary (Fig. 1). In mammals, that question has been extended to: How are testes and ovaries linked to the presence or absence of the Y chromosome?

This article reviews the cellular and morphological changes that take place during early development of the gonads and the underlying molecular events that underpin these changes. Many of the key regulatory genes have been identified in recent years, providing the challenge of discovering how they fit into the comprehensive network of gene activity and regulation that makes males and females.

II. THE CHROMOSOMAL BASIS OF MAMMALIAN SEX DETERMINATION

In 1916, Bridges described the sex chromosomes of the fruit fly *Drosophila melanogaster*, ascribing the sex determining mechanism to the X:autosome ratio, i.e., 2:2 in females (XX), 1:2 in males (XY). When the human X and Y chromosomes were first described by Painter (177), it was initially thought that humans would have a similar mechanism. Another 30 years elapsed before the first sex chromosome aneuploid mammals were discovered, which overturned this hypothesis and conclusively demonstrated that mammalian sex determination is dependent on the Y chromosome. In humans, XXY individuals develop testes (103) and XO individuals develop ovaries (75). Consequently, if sex were determined by the X:autosome ratio, the reverse would have been true.

In the following three decades it became increasingly obvious that development of testes is associated with the presence of a single Y-linked gene locus, dubbed *TDF* (testis determining factor) in humans and *Tdy* in mice. For simplicity, we will refer to both as *TDY*. As in all genetic analysis, this understanding arose out of the examination of mutations both in human and mouse that led to varying degrees of sex reversal, i.e., the chromosomal sex does not correlate with the observed sex. However, sterility is also usually a consequence of sex-reversing mutations, and therefore, such cases are generally sporadic, making conventional pedigree-based positional mapping difficult or impossible. Identification of TDY therefore had to rely on the study of sporadic cases of sex reversal.

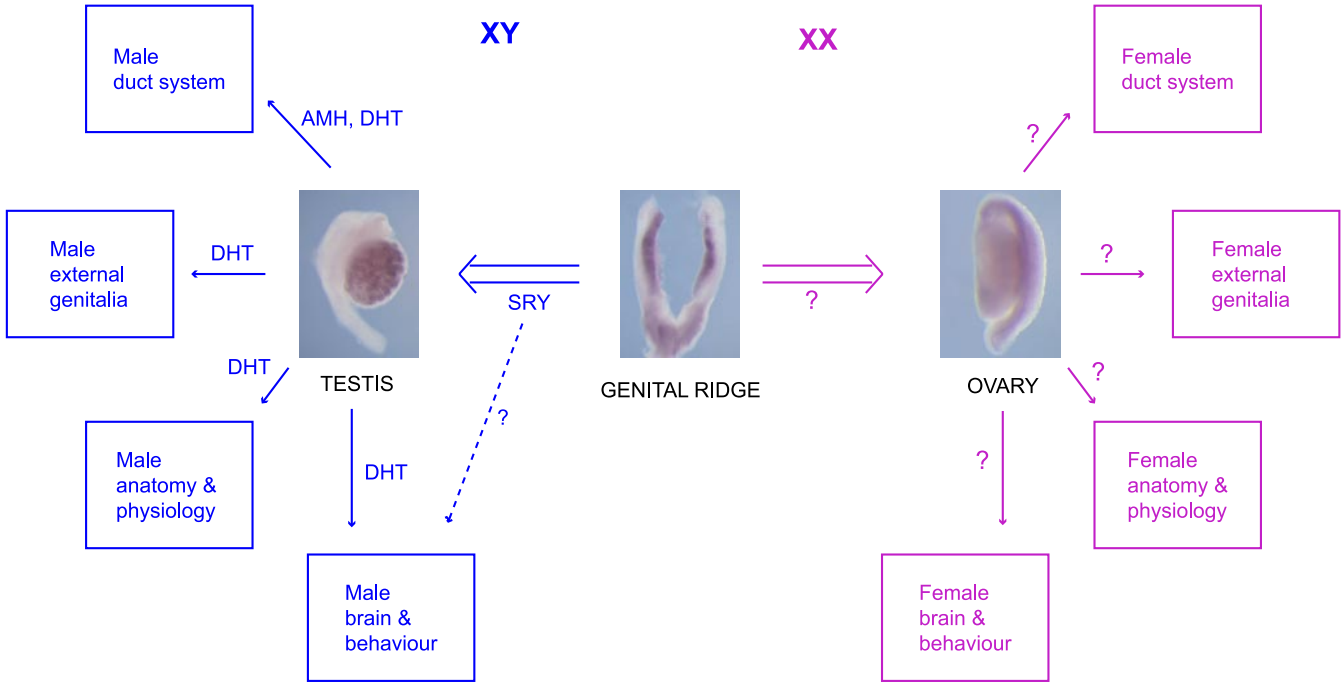

FIG. 1. Schematic representation of the development of sexual phenotype in mammals. The differentiation of the bipotential genital ridge into a testis requires the Y-encoded gene *Sry*. Subsequently, hormones such as androgens and anti-Müllerian hormone (AMH) produced by the developing testis direct the development of all secondary sexual differentiation. In the absence of *Sry*, all sexual differentiation follows the female pathway. DHT, 5α-dihyrotestosterone.

XX sex reversal often occurs in humans through the transfer of *TDY* onto the X chromosome due to an illegitimate recombination between the X and Y in male meiosis. Females normally have an identical pair of X chromosomes that can recombine during meiosis along their entire length in a similar fashion to autosomal pairs. In males, homology between the X and Y chromosome is restricted to a tiny region called the pseudoautosomal region (PAR), and it is in this region that pairing and recombination take place during male meiosis. In abnormal circumstances, pairing may extend into adjacent, nonhomologous regions, and an inappropriate exchange may occur that transfers Y-specific DNA onto the X chromosome. Regardless of the vast expanse of Y-unique DNA that *TDY* could occupy, humans, rather riskily, carry *TDY* less than 35 kb away from the PAR. This design fault results in a relatively high frequency of sex reversal, but ironically held the key to the discovery of the gene *SRY* that corresponds to the TDY locus (Fig. 2).

The testis-determining gene was eventually mapped and identified in humans by the analysis of four human XX males who carried a mere 60 kb of Y chromosomal DNA (178). A search for conserved sequences within the 60-kb region was initiated, and the gene *SRY* was rapidly isolated (215). Supporting evidence for *SRY* as the sought-after *TDY* came from the characterization of three XY females with no apparent cytogenetic abnormalities. One carried a frameshift mutation of *SRY* and the other two had single base substitutions in *SRY* (19, 105). The real proof was, however, derived from work in the mouse. First, *Sry* (the mouse ortholog) is deleted in a line of XY female mice (83). Second, *Sry* is expressed in the somatic component of the genital ridge at exactly the predicted time for testis determination, i.e., just before the appearance of testis cords (133). Finally, transgenic XX mice carrying a genomic fragment containing the *Sry* gene develop as males (132), which are sterile due to the adverse effect of two X chromosomes in spermatogenesis.

It is now widely accepted that the X and Y chromosome evolved from an ancestral pair of homologous autosomes. A simplified version of this hypothesis could be described as follows: a species ancestral to the mammalian line developed a dominant mutation that led to testis differentiation. It is likely that this mutation was in a gene already involved in the process of gonadal differentiation in some capacity. Carrying the mutation caused testis differentiation, and absence led to the development of ovaries. This moment defines the existence of the proto-X and proto-Y chromosomes, with the proto-Y carrying the newly formed *TDY* gene. In line with this hypothesis, there is evidence suggesting that *Sry* and the X-chromosomal gene *Sox3* have a common ancestral precursor (81, 82, 119). During the subsequent millennia, chromosomal rearrangements or translocations might place genes close

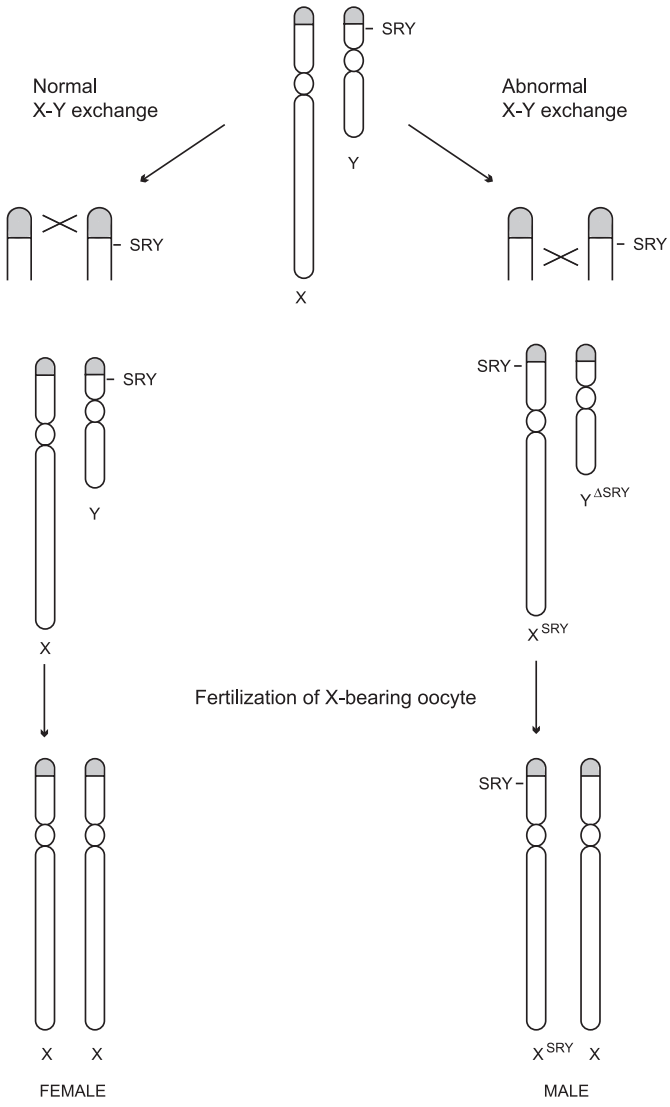

FIG. 2. Sex reversal in humans caused by abnormal X-Y exchange. During male meiosis, the X and Y chromosome align and genetically recombine at the pseudoautosomal region (PAR, marked in gray). Normal X-Y exchange (*left*) results, after fertilization of an X-bearing oocyte, in a normal XX female. Abnormal X-Y exchange (*right*), in which recombination occurs outside the PAR, leads to the transfer of some Y chromosomal DNA to the X chromosome. If this Y chromosomal DNA contains the testis-determining gene *SRY*, the fertilization of an X-bearing oocyte results in an XX male.

to *TDY* that were advantageous to males and/or disadvantageous to females. In this circumstance, recombination of such genes onto the proto-X chromosome would be deleterious for females and either have no consequences in the male or be advantageous. This would cause a suppression of recombination between the X and Y in a region around *TDY*. As time progressed, this nonrecombining region would spread as more sex-differential genes were accumulated and the proto-Y would drift in its genetic composition from the proto-X until the present X and Y chromosome were formed. Comparative genetics of

the sex chromosomes of various mammalian species support this oversimplified model. However, additions of autosomal material onto the X and Y followed by erosion of these sequences from the Y chromosome seem to have occurred many times in mammalian evolution so that, while all mammalian sex chromosomes share common features, their composition can be quite different (for a detailed review, see Ref. 79).

One might think that the Y chromosome would eventually lose all homology with the X. However, mice, humans, and probably many other mammalian species seem to have an essential requirement for the preservation of X and Y pairing for the process of meiosis and XY chromosomal segregation during spermatogenesis (37). Therefore, this opposing force appears to maintain the existence of a small region of homology between the X and Y.

III. MORPHOLOGY AND CELL BIOLOGY OF THE DEVELOPING GONAD

Much of the biology described in the following sections is based on work conducted on developing mouse embryos. The mouse is, by far, the most studied and the best-characterized model of mammalian sex determination. It is assumed that events in the human embryo follow the same basic pattern, even if there are differences in timing and anatomy. What little information exists on the human supports this notion. However, variations in mammalian sexual differentiation are known to exist, and this needs to be kept in mind when extrapolating from one species to another.

A. The Bipotential Gonad

The development of the gonads can be divided into two phases. The initial phase is characterized by the emergence of the so-called indifferent, bipotential gonad, or genital ridge, which is identical in males and females. The cell lines that comprise it are bipotential, being able to adopt either the male or female fate. The second phase is the development of a testis or an ovary, which, as we discuss in detail later, is triggered solely by the expression and proper function of the testis-determining gene *Sry*.

The indifferent gonads arise as paired structures within the intermediate mesoderm, which lies on either side of the embryo filling much of the coelomic cavity between the limb buds during the first half of development. Within this region, three segments comprising the urogenital ridge are distinguished from anterior to posterior: *1*) the pronephros, which includes the adrenal primordium near its caudal end; *2*) the mesonephros, the central region from which the gonad arises; and *3*) the metanephros, the most posterior region from which the kidney forms.

The gonads emerge on the ventromedial surface of the mesonephros at ~10.5 days post coitum (dpc). Cells that delaminate from the coelomic epithelium seem to provide one source of cells for the growing genital ridges, while recruitment of underlying cells from the mesonephros to the epithelial population also augments the cell population in the gonadal primordium in males (see below). From the earliest stages of gonad development, the mesonephric tubules can be seen to form continuous bridges to the epithelial cells of the gonad in male and female genital ridges (117). These structures, which have been reported previously in electron microscopy studies (238), are obvious by confocal imaging of whole gonads stained with antibodies against laminin and E-cadherin (Fig. 3). No function for these tubule connections has yet been defined.

The early mammalian gonad is an undifferentiated primordium composed of bipotential precursor cells that can follow one of two possible fates. Precursors for supporting cells (so named for their role in sustaining and nourishing germ cells in both sexes) and steroid-secreting cells are believed to be present in the early gonad (156). Supporting cell precursors continue to delaminate from the coelomic epithelium until ~11.5 dpc (116), as shown by dye-marking experiments.

Several lines of evidence indicate that the supporting cell precursors can develop into either testis-specific Sertoli cells or ovary-specific follicle (granulosa) cells. In mosaic gonads, consisting of a mixture of XX and XY cells, small numbers of XX cells have been seen to develop as Sertoli cells (179), and XY cells have been seen to

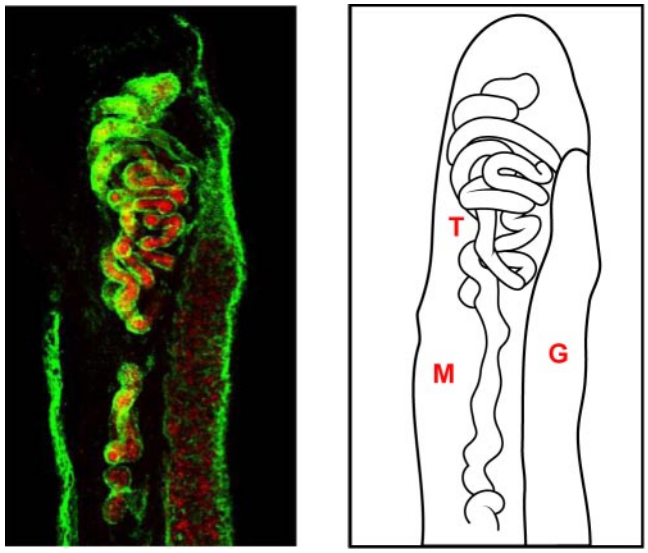

FIG. 3. Mesonephric tubules in the 11.5 dpc mouse urogenital ridge. Whole-mount immunofluorescence with antibodies to laminin (green) and E-cadherin (red) and confocal imaging were used to visualize the mesonephric tubules and the partially epithelialized cells of the gonadal primordium. G, gonadal primordium; M, mesonephros; T, mesonephric tubules.

develop as granulosa cells (38, 180). In addition, transgenic mice that express a reporter protein (green fluorescent protein, GFP) under the control of the *Sry* promoter show expression not only in Sertoli cells in XY gonads, but also in granulosa cells in an XX gonad (4). Evidence for the origin of the steroidogenic lineage is less clear at present, but the steroid-secreting Leydig cells in the testis and theca cells in the ovary, which engage in many common steroid pathways, probably also derive from a single precursor. In contrast, there is good evidence to suggest that other characteristic cell types in the gonad are recruited differently in the testis versus the ovary (see sect. III*D*).

B. Development and Differentiation of the Ductal System

In mammals, the primordia for both male and female duct systems are initially present in the mesonephroi. The Wolffian (or mesonephric) ducts are the progenitors of the male duct system and first appear in the mouse in short, transient segments within the pronephros, then as a stable continuous tube along the length of the urogenital ridge, adjoining the cloaca at its caudal end (45). The antecedent of the female ductal system, the Müllerian (or paramesonephric) duct, forms by invagination of a tube from the surface epithelium of the mesonephros. This tube runs parallel to the Wolffian duct in both male and female embryos. Only one of the two duct systems will normally develop further in mammals, depending on whether differentiation of a testis or ovary has begun (Fig. 4). Posteriorly, the kidney is formed by an inductive interaction between the ureteric bud, branching of the Wolffian duct, and the metanephric mesenchyme. Experimental work in chickens suggests that mesonephric differentiation and subsequent gonad development is also dependent on a similar inductive interaction between the Wolffian duct and the intermediate mesoderm (22, 73). The mesonephric tubules (Fig. 3) form shortly after the appearance of the Wolffian duct and extend through the mesenchyme of the mesonephros toward the coelomic surface by a process of condensation that resembles branching morphogenesis in the kidney. The mesonephric tubules may play a role in the development of both the adrenal gland and the gonad either through signaling to the surrounding regions or by the direct contribution of cells to the forming organs.

C. Origin of Primordial Germ Cells and Their Migration to the Genital Ridge

The primordial germ cells (PGCs) do not arise within the genital ridge or the mesonephros but migrate from an entirely separate source. Due to their characteristic property of positive staining with alkaline phosphatase, it is possible to trace their origin to the base of the allantois at the posterior end of the primitive streak. Results of cell labeling experiments suggest that a population of ~45 cells is allocated to the germ line at 7 dpc in the mouse (136). At 6–6.5 dpc, the precursors of the PGCs can be found in the epiblast close to the extraembryonic ectoderm, but are evidently not yet restricted to a germ cell fate because they can also form extraembryonic mesoderm.

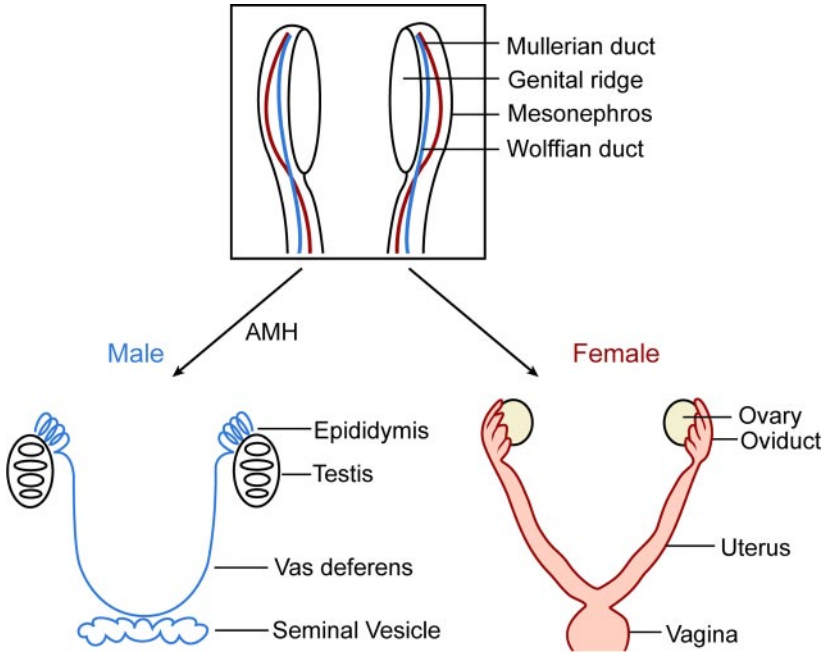

FIG. 4. Development and differentiation of the genital duct system. Both Müllerian and Wolffian ducts are present at the bipotential stage. In males, the Müllerian ducts degenerate under the influence of AMH secreted by the testicular Sertoli cells, whereas the Wolffian ducts differentiate into epididymides, vasa deferentia, and seminal vesicles under the control of androgens produced by Leydig cells. In females, the Wolffian duct regresses and the Müllerian duct differentiates into oviduct, uterus, and upper vagina.

It is interesting to note that the PGCs continue to express genes that are characteristically associated with the maintenance of an undifferentiated pluripotent state. For example, the transcription factor OCT4 and alkaline phosphatase are both expressed in the embryonic inner cell mass (ICM) during the very earliest stages of embryogenesis at a time when those cells are practically totipotent. Cells of the ICM are the source of embryonic stem cells (ES cells), which are capable of proliferating indefinitely and contributing cells to all tissues. The loss of *Oct4* and alkaline phosphatase expression correlates with lineage restriction as different regions of the embryo become determined into the primary germ layers. However, the PGCs continue to express these markers and embryonic germ (EG) cell lines have now been derived from them, which have properties similar to ES cells and can be harvested from the embryo as late as their entry into the genital ridge (150).

When PGCs are first seen in the mouse at 7 dpc, they are in the region of the forming hindgut. As development proceeds, the hindgut invaginates and the germ cells are swept into the embryo. Although germ cells have the capacity for active migration, this early stage is likely to be a passive process because the appearance of PGCs at this time suggests that they are nonmotile. By 9.5 dpc, PGCs begin to leave the hindgut and pass into the forming urogenital ridges, which are in close proximity at this time. As development proceeds, the hindgut descends into the coelomic cavity and PGCs arriving later must migrate through the dorsal mesentery before entering the developing gonads (Fig. 5). Survival of the PGCs during migration is dependent on an interaction between the tyrosine kinase receptor c-KIT, which is present on the surface of PGCs, and its ligand, stem cell factor (SCF), which is produced by the surrounding tissues (reviewed in Ref. 17). During migration the PGCs also undergo several rounds of cell division to achieve a population of ~3,000 cells by 11.5 dpc, when almost all the PGCs have arrived at their destination.

Once inside the genital ridge, the germ cells lose their motility and begin to aggregate with one another. They continue to proliferate within the indifferent gonad and maintain their bipotentiality until 13 dpc, whereupon germ cells within the male gonad become enclosed within the forming testis cords and enter mitotic arrest as T1 prospermatogonia. In the female, proliferation continues for a short while longer before the germ cells enter meiosis at 13.5 dpc.

PGCs thus have the potential to develop either as meiotic oocytes, progressing through the first meiotic prophase and arresting in diplotene just after birth, or as prospermatogonia, mitotically arrested in G_1/G_0 until a few days after birth, when they resume proliferation (90, 151). This developmental switch, which has occurred by 13.5 dpc, is dependent on the sex of the somatic cells in the gonad, rather than the chromosomal sex of the PGCs: XY PGCs can develop as oocytes in female embryos, and XX PGCs can develop as prospermatogonia in male embryos (74, 179).

D. Testis Differentiation

Testis differentiation is induced by the expression of *Sry* in a subset of somatic cells that are induced to differentiate into Sertoli cells. Sertoli cells are believed to act as the organizing center of the male gonad and orchestrate the differentiation of all other cell types. In the following sections we summarize what is known about the origin and differentiation of the various testis-specific cell types (Fig. 6).

1. Sertoli cells

Sertoli cells are somatic cells that associate with germ cells and nurture their development into sperm. They are the first cell type known to differentiate within the gonad from bipotential precursors of the supporting cell lineage and are therefore the first indicator that the gonad has passed from the indifferent stage into testis development. In situ hybridization (133) and RNase protection studies (84) showed that *Sry* is expressed in the gonad at 11.5 dpc and that this expression is associated with the somatic cells of the genital ridge and not the germ cells (133, 199). These cells become positive for *Sry* mRNA only after delaminating from the coelomic epithelium, indicating that *Sry* is not the cause of this delamination (35). However, definitive evidence that *Sry* is ex-

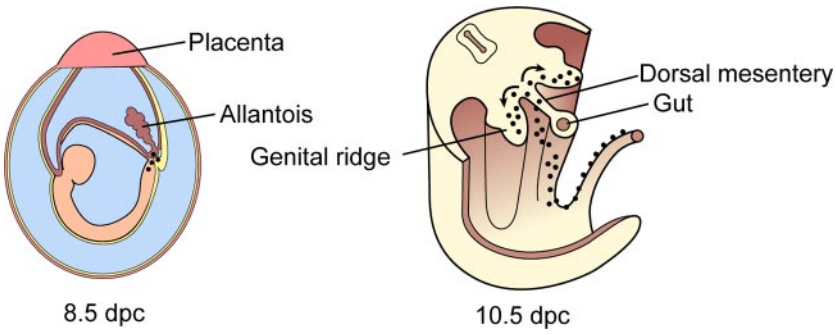

FIG. 5. The migratory pathway of primordial germ cells. Schematic representation of the localization of PGCs (black dots) at the base of the allantois around the hindgut pocket in an 8.5 dpc mouse embryo (*left*) and their migration along the hindgut, dorsal mesentery, and into the genital ridges in a 10.5 dpc embryo (*right*).

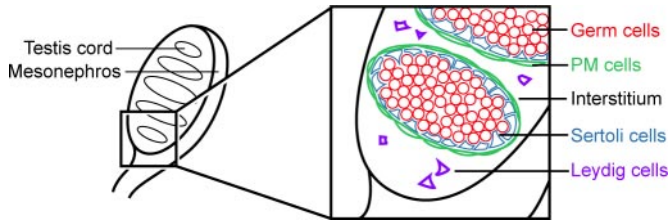

FIG. 6. Structure of the early fetal testis. Schematic diagram of a 13.5 dpc mouse testis showing the developing testis cords (*left*). The testis is adjacent to the mesonephros, which contains the Wolffian duct. Enlargement of the area demarcated by the rectangle shows the cellular organization of the testis (*right*). Clusters of germ cells (red) are enclosed by the supporting Sertoli cells (blue) and a layer of peritubular myoid cells (PM cells, green). Steroidogenic Leydig cells (purple) reside in the interstitium between the testis cords.

clusively expressed in Sertoli cells, or more accurately the pre-Sertoli cells (see below), were hampered for many years by the lack of a molecular tool to detect endogenous mouse SRY expression in situ. Transgenic mouse models, expressing either GFP (4) or an epitope-tagged SRY (209), under the control of the *Sry* promoter, suggested that *Sry* expression is restricted to the Sertoli cell lineage. However, the correctness of the temporal and spatial expression pattern of these transgenes cannot be guaranteed, because the regulatory regions of endogenous *Sry* have not been characterized. Furthermore, epitope-tagging might affect posttranscriptional processing such as mRNA and protein stability and translational efficiency. More direct studies recently became possible through the generation of a mouse SRY antibody and demonstration that the subset of somatic cells that expresses SRY almost immediately start to coexpress SOX9, which in turn is a reliable lineage marker of developing Sertoli cells (245).

The differentiation from pre-Sertoli cells into Sertoli cells is marked by the polarization of the cells when they form epithelial aggregates that assemble into testis cords. Concurrently there is a change in the expression of certain extracellular matrix proteins; desmin is downregulated, whereas cytokeratins are upregulated (78). On the basis of these findings, pre-Sertoli cells are defined as nonpolarized, dispersed somatic cells that express *Sry* and/or *Sox9*, whereas a Sertoli cell is polarized, resides within the testis cord structure, and expresses *Sox9* (Fig. 7).

In aggregation chimeras made between mouse embryos of an XX and XY genotype, the proportion of XX to XY cells is, on average, 50:50 in all the tissues of the body. Within the testes of such mice, all lineages with the exception of Sertoli cells were found in a 50:50 ratio. However, Sertoli cells were >90% XY, indicating a strong bias in this cell lineage for the presence of the Y chromosome (179). These experiments indicate that Sertoli cells are the only cell type within the developing testis that requires the cell-autonomous expression of *Sry*. Nevertheless, they also imply that *Sry* is not necessary for differentiation of all Sertoli cells. In these chimeric experiments, a small percentage of XX cells were recruited to develop into Sertoli cells. In vitro cell mixing experiments (245) have demonstrated that prostaglandin D_2 (PGD_2), produced and secreted by Sertoli cells, is necessary and sufficient to recruit cells that do not express *Sry* (XX cells in the above-mentioned experiments) to express *Sox9* and differentiate into Sertoli cells (Fig. 8). Other experiments have shown that the number of Sertoli cells has to reach a certain threshold to guarantee testis development (36, 179). The paracrine signaling via PGD_2 could function

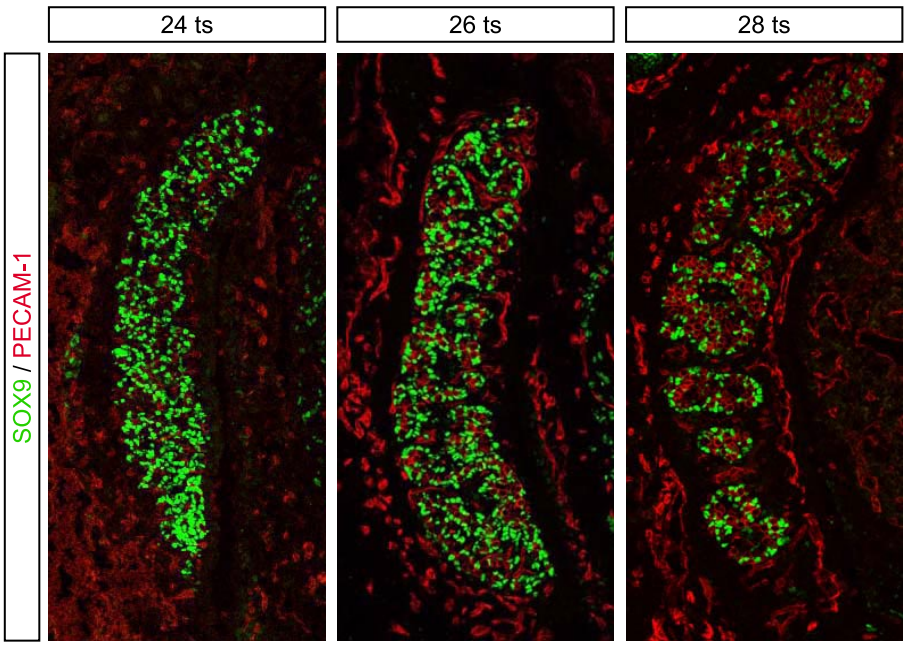

FIG. 7. Differentiation of pre-Sertoli cells into Sertoli cells. Nonpolarized, dispersed somatic cells visualized by SOX9 immunofluorescence (green) represent pre-Sertoli cells at the 24-tail somite (ts) stage. A few hours later, by 28 ts, these cells become polarized, forming epithelial aggregates that assemble into testis cords; at this stage they are referred to as Sertoli cells. PECAM-1 counterstaining (red) marks PGCs and endothelial cells.

as a backup mechanism in case of impaired *Sry* function, to ensure the Sertoli cell number threshold is reached and male sexual differentiation will commence.

By varying the ratio of XX to XY cells in XX-XY chimeras, it has been possible to establish that a minimum of ~20% of the supporting cell lineage must be XY, and therefore potentially expressing SRY, in order for full testis development to proceed (36, 184). This means either that up to 80% of non-SRY-expressing supporting cell precursors can be induced to differentiate as Sertoli cells through paracrine signaling mechanisms, or that 20% of the normal number of Sertoli cells is able to secrete sufficient levels of signaling molecules to ensure complete male differentiation of other lineages in the testis.

In parallel to the differentiation of Sertoli cells, the gonad increases remarkably in size due to increased proliferation and migration of cells from the adjacent mesonephros. These processes occur only in males after the onset of *Sry* expression (46, 146, 205). The immigrating mesonephric cells give rise to peritubular myoid cells, endothelial cells that form the male-specific vasculature, and, at least in part, to steroidogenic Leydig cells. This migration is most likely induced by secreted factors expressed under the direct or indirect control of *Sry*. Several factors have been implicated such as neurotropin-3 (NT-3; Ref. 55), hepatocyte growth factor (HGF; Ref. 196), and platelet-derived growth factor (PDGF; Ref. 217). However, all data were obtained in vitro or in ex vivo gonad cultures by using purified factors or inhibitors of their signaling pathways; it is still not known whether these factors play a role in vivo. Moreover, it is unclear whether a single factor induces the migration of one or more precursor cell types that subsequently differentiate into the different testicular cell populations by direct interaction with gonadal cells, or whether several factors are each responsible for the migration of different cell precursors. Experiments to date indicate that the first mechanism might be functioning, because interference with any of the pathways studied so far resulted in the blockage of migration of all cells, not just a subset of cells.

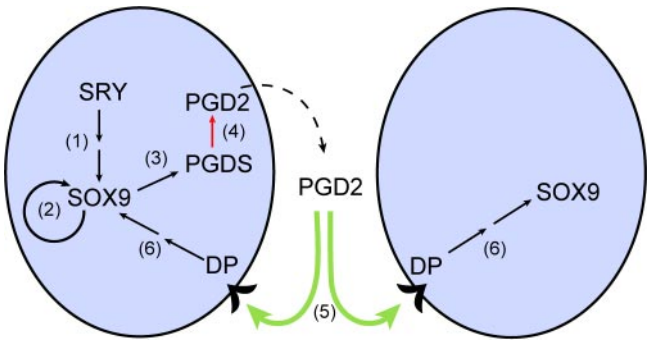

FIG. 8. Model for cell-autonomous and prostaglandin-mediated upregulation of *Sox9* in pre-Sertoli cells. *Sry* induces *Sox9* cell-autonomously either via a direct or indirect regulatory mechanism (*1*). Subsequently, *Sox9* maintains its own expression in an autoregulatory loop (*2*). In addition, *Sry* and/or *Sox9* serve to upregulate *Pgds* (*3*), which leads to prostaglandin D_2 (PGD_2) synthesis (*4*) and secretion. PGD_2 can act by binding to its receptor DP (*5*), to upregulate *Sox9* expression in a paracrine, and possibly also an autocrine manner (*6*). Thus cells that do not express *Sry* or fail to reach a threshold of *Sry* expression can be induced to upregulate *Sox9* and differentiate as Sertoli cells. [Adapted from Smith et al. (216).]

2. Peritubular myoid cells

One of the three cell types that migrate from the mesonephros into the male gonad is the peritubular myoid (PM) cell. These cells form a single layer of flattened cells surrounding the Sertoli cells, circumscribing the testis cords. They are thought to have two main functions: *1*) to contribute structurally to the formation of the testis cords in conjunction with Sertoli cells, a function that will be discussed in more detail later; and *2*) to promote the movement of mature sperm through the seminiferous tubules of the adult testis for export to the seminal vesicles, a function mediated by their smooth muscle-like character. PM cells express α-smooth muscle actin (αSma) and desmin and contract in vitro after $PGF_{2\alpha}$ treatment (234). However, attempts to find specific marker genes for this cell type have not yet succeeded (106). PM cells represent the only cell type in the testis so far for which no counterpart can be identified in the ovary. This might be due to their origin from immigrating cells from the mesonephros, which only occurs in an XY gonad after the expression of *Sry* (46, 146).

3. Testis cord formation

Under the light microscope, the first signs of testicular differentiation appear in the mouse at 12.5 dpc with the formation of cylindrical cords, the precursors of the adult spermatogenic tubules. They are composed of clusters of germ cells enclosed by a layer of Sertoli cells, which is in turn surrounded by a layer of PM cells (Figs. 7 and 9). Under the electron microscope, Sertoli cells can be recognized before the formation of testis cords, and it is argued therefore that their differentiation precedes their aggregation (112). Interestingly, testis cords can form in the genetic or pharmacologically induced absence of germ cells (149, 155), demonstrating a negligible role of germ cells in this process. On the other hand, in XY gonad explant cultures without adjacent mesonephroi and consequently lacking PM cells, the formation of the cords is disrupted, showing that in addition to the Sertoli cells, PM cells are necessary (32). Surprisingly, similar gonad explant culture experiments also suggested that PM cells, or at least immigrating mesonephric cells, are not only necessary, but maybe also sufficient to induce Sertoli cell differentiation and cord formation. In these experiments, XX gonads were placed between a mesonephros and an XY gonad, and cells were induced to migrate from the mesonephros through the XX gonad, thus inducing Sertoli

cell-specific marker expression and organization of cord structures (229). Subsequently, PM cells and the Sertoli cells collaboratively induce the deposition of a basal lamina between their respective layers, thus defining the boundary between the testis cords and the interstitial tissue. Clearly, derivation of a reliable molecular marker for these cells is a priority if we are to understand the origin and functions of the PM cell lineage in more detail.

4. Leydig cells

Within the second compartment of the testis, the interstitium, steroidogenic Leydig cells differentiate (Fig. 9). They were first described by Franz Leydig in 1850 (138), but it took over 50 years before Bouin and Ancel demonstrated, by working with cryptorchid animals, that these cells secrete a hormone that plays a role in establishing and maintaining the secondary male sex characteristics (25). Leydig cells often lie in clusters close to blood vessels, in line with their steroidogenic role. In mammals there are two types of Leydig cells. The fetal Leydig cells originate, at least in part, in the mesonephros, and are responsible for the production of androgen for the fetal masculinization; these cells probably degenerate postnatally. The adult Leydig cells, which differentiate after birth, appear to be unrelated to their fetal counterparts. Studies indicated that they arise from undifferentiated precursor cells that are part of the mesenchymal cells of the interstitium (60, 87). The origin and roles of Leydig cells are discussed comprehensively elsewhere (186).

5. Vascular and other interstitial cells

Although Leydig cells are often considered the main component of the testicular interstitium, probably because of their essential and obvious male-specific endocrine roles, several other interstitial cell types can be found. These include endothelial cells, fibroblasts, and blood-derived cells such as macrophages, lymphocytes, plasma cells, monocytes, and mast cells. Endothelial cells, alongside PM and Leydig cells, represent a third cell type that migrates into the testis from the mesonephros (146). They form the male-specific vasculature with the prominent coelomic vessel on the surface of the gonad and side branches in between the testis cords. Inhibitor experiments and mutant analysis so far suggest that the formation of the vasculature and the testis cords are intimately interrelated; there is no example, to our knowledge, where one of the two processes was impaired without affecting the other. It will be interesting to identify the factors that carry out this cross-talk between endothelial cells and Sertoli and/or PM cells.

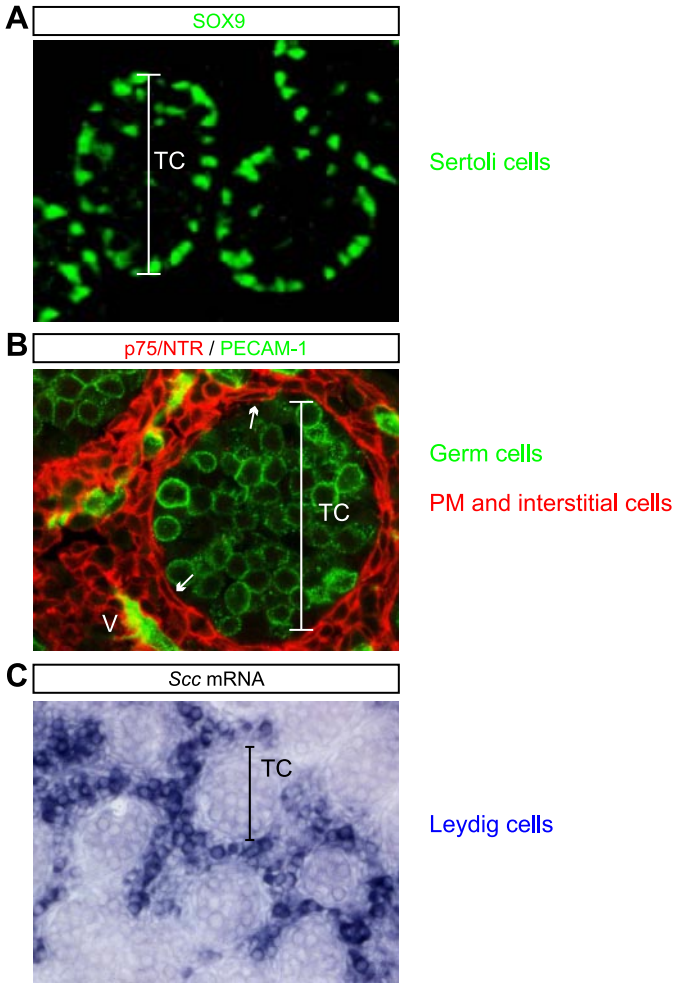

FIG. 9. Visualization of testicular cell types. *A*: immunofluorescence of sections of 14.5 dpc mouse testis with an antibody to SOX9 marking the Sertoli cells (green). *B*: immunofluorescence of sections of 14.5 dpc mouse testis with antibodies to PECAM-1 (green) marking PGCs enclosed in testis cords (TC), and p75/NTR (red) staining peritubular myoid cells (white arrows) and interstitial cells. Endothelial cells (yellow) are positive for both cell surface molecule. PM cells, peritubular myoid cells; V, vasculature. *C*: section in situ hybridization of 14.5 dpc mouse testis for cholesterol side-chain cleavage enzyme (Scc) expressed by interstitial Leydig cells (dark purple).

E. Differentiation of Primordial Germ Cells

Once the testis cords have formed, the mitotically dividing PGCs block at the G_0/G_1 phase of mitosis and differentiate into T1-prospermatogonia, a state in which they will remain until after birth. In cultures in which the Sertoli cells are forced to disaggregate by the addition of cAMP analogs, the germ cells nevertheless still arrest as T1-prospermatogonia, arguing against a connection between enclosure of the germ cells and their mitotic arrest (225).

Germ cells seem to play a more active role in ovary development than they do in testis development. In the absence of PGCs, supporting cells in the ovary differentiate into prefollicle cells that aggregate into mesenchy-

mal condensations, but these eventually degenerate, leaving only stromal tissue. The involvement of germ cells in the differentiation and maintenance of the male supporting cell lineage is less obvious, as germ cells are required neither for the differentiation of Sertoli cells nor for the assembly of testis cords (reviewed in Ref. 149). When germ cells migrate into ectopic sites such as the adrenal gland, they will develop as oocytes even in male embryos, entering meiosis with apparently normal timing (256). This can be simulated in culture using isolated PGCs in lung aggregates (152). It has been proposed that PGCs enter meiosis driven by an intrinsic clock and that an as yet uncharacterized signal produced by the somatic cells in a male genital ridge inhibits PGCs from entering meiosis, arresting them in G_1/G_0, and directing them towards spermatogenesis (64, 152). PGCs in male genital ridges can be rescued from this signal if they are removed from the genital ridges at 11.5 dpc and will develop as oocytes in lung aggregates. However, by 12.5 dpc, PGCs isolated from male genital ridges are committed to spermatogenesis (1, 152).

An alternative view is suggested by the observation that entry into meiosis in the female embryo occurs in an anterior to posterior wave, a situation that arguably might not occur under the action of an intrinsic clock (256). In support of the possible existence of a meiosis-inducing substance, Byskov and Saxen (41) showed, using cocultures of fetal testes and ovaries, that nonmeiotic germ cells within the testes were induced to enter meiosis by close apposition with ovarian tissue. A dose-dependency was observed when conditioned medium from cultures of adult ovaries and testes were used to trigger meiosis in embryonic testes (39, 40). These observations suggest that in the embryonic testes either this meiosis-inducing factor does not exist or, in addition, a meiosis-inhibiting factor is produced.

Recent studies have revealed the signaling events that regulate germ cell entry into meiosis in mouse fetal gonads. Retinoic acid (RA) produced within the mesonephros functions as an inducer of entry into meiosis (28a, 133a). PGCs in male gonadal explant cultures treated with exogenous RA start to express meiosis markers such as *Stra8*, *Scp3*, and *Dmc1*, whereas PGCs in female explant cultures treated with inhibitors of RA signaling do not enter meiosis but instead continue to express the pluripotency marker *Oct4*. Fetal adrenal and lung also express high levels of enzymes that produce RA, providing a ready explanation for the ability of these tissues to stimulate entry of PGCs into meiosis. Male germ cells are protected from the effects of RA by their location within the testis cords. Sertoli cells, which surround PGCs in the testis cords, express CYP26B1, an enzyme that catabolyzes RA and is therefore the male-specific meiosis-inhibiting factor (28a).

F. Ovary Differentiation

The ovary has two main functions: *1*) the production of steroid hormones and *2*) the generation of mature oocytes that are capable of being fertilized and developing into an embryo. The functional unit of the ovary is the ovarian follicle in which the oocytes mature, surrounded by granulosa and thecal cells (Fig. 10). In contrast to the testis, in which the functional unit, the testis cord, forms at around 12 dpc, the ovarian follicles commence differentiation only after birth.

1. Early somatic differentiation

Unlike males, where a rapid burst of testis differentiation is triggered by the expression of *Sry*, in females, the fetal gonad appears inert for several days in the mouse. However, female-specific gene expression has been reported as early as 11.5 dpc (28, 33, 111, 169, 252). Furthermore, detailed histological analysis revealed that there is a phase from around 13.5 to 15.5 dpc, in which the poorly differentiated structure undergoes remodeling (Fig. 10). The presumptive oocytes develop as interconnected cysts that are linked by cytoplasmic bridges (187). There is also a high degree of vascularization, with a dense network of small vessels that become visible only by using molecular markers (33). These vessels demarcate strings of germ cells, also known as ovigerous cords

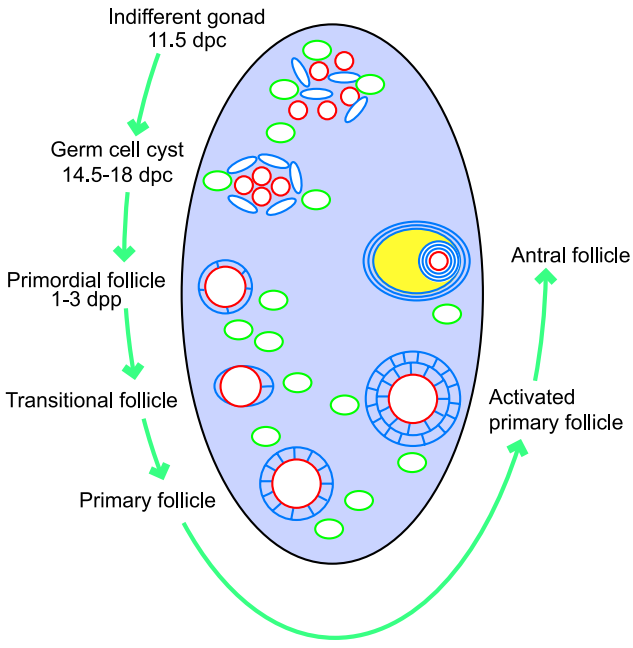

FIG. 10. Ovary and follicle development and differentiation. Schematic representation of the stages of cellular organization in the fetal and postnatal mouse ovary, leading to primordial, primary, and antral follicle formation. Oocytes are shown in red, supporting pregranulosa cells in blue, steroidogenic thecal cells in green, and antral fluid in yellow. Stages of thecal development are omitted for simplicity.

(130, 172). The function of this vascularization is not known. For the male-specific vasculature it was first hypothesized that it is more prominent compared with the female because it might be necessary for the transport of steroid hormones produced in the testis, but not in the ovary at this stage of development. Closer examination revealed that this male-specific vasculature is comprised of arteries rather than veins (30). This could suggest that the vasculature in males and females might serve the same function, that is, to deliver exogenous growth factors to the somatic and/or germ cells, and that the differences in patterning are secondary effects due to the different morphological organization (testis cords vs. ovigerous cords).

2. Formation of primordial follicles

Within the first 3 days after birth, a rapid reorganization of the ovarian morphology becomes obvious. The intercellular bridges between oocytes within the ovigerous cords break down, and single oocytes become closely surrounded by a somatic epithelial monolayer of flattened, squamous pregranulosa cells. These definitive primordial follicles are separated from the somatic compartment by a basement membrane surrounding the pregranulosa cells (Fig. 10). At the same time, there is a reorganization of the ovary into morphological compartments, the cortex, where the primordial follicles reside, and the medulla. During the formation of the primordial follicles, high levels of oocyte apoptosis occur (13, 24). The signals for this programmed cell death are not known, but it is likely to be a combination of intercellular signals (every oocyte needs to be surrounded by a sufficient number of pregranulosa cells) and intracellular signals (the oocyte has to be fit enough to progress further). This massive cell death limits the number of primordial follicles. Proliferation of female germ cells only takes place during embryogenesis, in contrast to the continuous proliferation of male germ cells; it has been long thought that the female has a finite number of primordial follicles, which, together with the rate of depletion of this pool, determines the female reproductive life span. This dogma has been challenged by recent reports of germ line stem cells (GCS) resident within the bone marrow. This pool of stem cells may replenish the ovary with new oocytes (108, 109), an exciting possibility that has yet to be substantiated.

During the formation of primary follicles the pregranulosa cells become cuboidal and start to proliferate, the oocyte increases in size, produces the zona pellucida, an extracellular glycoprotein matrix deposit between the oocyte and the granulosa cells, and subsequently the follicle becomes surrounded by thecal cells. These processes are controlled by intragonadal factors that initiate the growth and extragonadal factors synchronizing granulosa and thecal cell function at later stages of folliculogenesis. Other primordial follicles remain quiescent until later so that there is a continuous production of preovulatory follicles, and follicles of each stage (primordial, primary, transitional, secondary, and antral) can be found at any given time. However, not all follicles eventually ovulate successfully. Many are lost during folliculogenesis via atresia, a degenerative process involving loss of granulosa cells by apoptosis and subsequently the loss of the oocyte.

IV. MOLECULAR PATHWAYS OF SEX DETERMINATION AND GONAD DEVELOPMENT

A. Genes Important for Formation of the Bipotential Gonad

Mutation analysis in mice and humans resulted in the identification of several genes that are important for the initial formation of the indifferent genital ridge (Fig. 11 and Table 1). These include Wilms' tumor suppressor 1 (*Wt1*; Refs. 85, 134), steroidogenic factor 1 (*Sf1*; Ref. 143), empty-spiracles homeobox gene 2 (*Emx2*; Ref. 158), the member of the polycomb group *M33* (121), and Lim homeobox gene 9 (*Lhx9*; Ref. 20).

1. Wilms' tumor suppressor 1

Wt1 is expressed widely throughout the urogenital ridge, in the mesonephros, the kidney, and the gonad (6). It is believed to mediate both the outgrowth of the ureteric bud and the response of the metanephric mesenchyme to the growth of the ureteric bud during kidney development (161). In $Wt1^{-/-}$ mutants, the cranial

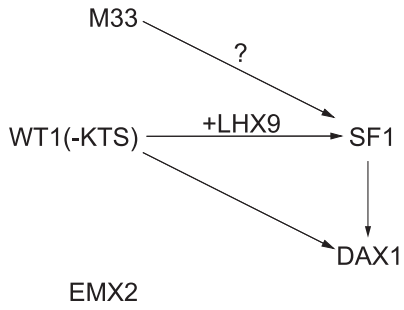

FIG. 11. Postulated molecular pathway leading to the formation of the bipotential genital ridge. Several molecules have been implicated in the formation of the bipotential gonad, and a regulatory network has started to emerge. The -KTS splice form of the Wilms' tumor suppressor WT1 regulates the expression of *Dax1*, and, together with LHX9, is responsible for the upregulation of *Sf1* expression. SF1 in turn also plays a role in the regulation of *Dax1* expression. In contrast, M33 and EMX2 have been difficult to fit into the regulatory scheme.

TABLE 1. *Genes implicated in sexual development in mammals*

Gene	Protein Function	Gonad Phenotype of Null Mice	Human Syndrome	Reference Nos.
		Bipotential gonad		
Wt1	Transcription factor	Blockage in genital ridge development	Denys-Drash, WAGR, Frasier syndrome	71, 134
Sf1	Nuclear receptor	Blockage in genital ridge development	Embryonic testicular regression syndrome	143, 200, 214
Lhx9	Transcription factor	Blockage in genital ridge development	*	20
Emx2	Transcription factor	Blockage in genital ridge development	*	158
M33	Transcription factor	Gonadal dysgenesis	*	120
		Testis-determining pathway		
Gata4/Fog2	Transcription/cofactor	Reduced *Sry* levels, XY sex reversal	*	227
Sry	Transcription factor	XY sex reversal	XY sex reversal (LOF); XX sex reversal (GOF)	19, 132
Sox9	Transcription factor	XY sex reversal	Campomelic dysplasia, XX sex reversal (GOF)	10, 21, 48, 76, 230, 241, 242
Sox8	Transcription factor	XY sex reversal in combination with partial loss of *Sox9* function	*	48
Fgf9	Signaling molecule	XY sex reversal	*	51, 206
Dax1	Nuclear receptor	Impaired testis cord formation and spermatogenesis	Hypogonadism	26, 153, 154, 163
Pod1	Transcription factor	XY sex reversal	*	54
Dhh	Signaling molecule	Impaired differentiation of Leydig and PM cells	XY gonadal dysgenesis	23, 43, 44, 49, 189, 237
Pgdra	Receptor	Reduction in mesonephric cell migration	*	31
Pgds	Enzyme	No phenotype	*	1, 145, 245
Arx	Transcription factor	Abnormal testicular differentiation	X-linked lissencephaly with abnormal genitalia	118, 127
Atrx	Helicase	ND	ATRX syndrome	226
Insl3	Signaling factor	Blockage of testicular descent	Cryptorchidism	2, 115, 168, 261
Lgr8	Receptor	Blockage of testicular descent	Cryptorchidism	2, 72, 115
Hoxa10	Transcription factor	Blockage of testicular descent	Cryptorchidism	97, 102
Hoxal1	Transcription factor	Blockage of testicular descent	Cryptorchidism	97, 102
Amh	Hormone	No Müllerian duct degeneration	Persistent Müllerian duct syndrome	14, 15, 100
Misrl1	Receptor	No Müllerian duct degeneration	Persistent Müllerian duct syndrome	94, 100
Pax2	Transcription factor	Dysgenesis of mesonephric tubules	*	45
Lim1	Transcription factor	Agenesis of Wolffian and Müllerian ducts	*	128, 129
Dmrt1	Transcription factor	Loss of Sertoli and germ cells	XY female†	194
		Ovary-determining pathway		
Wnt4	Signaling molecule	Müllerian duct agenesis, testosterone synthesis, and coelomic vessel formation	XY female (GOF)	89, 239
FoxL2	Transcription factor	Premature ovarian failure	BPES	53, 175, 207, 236
Dax1	Nuclear receptor	XY sex reversal (GOF)	XY sex reversal (GOF)	110, 163, 223, 257

* No mutations in human sexual disorders identified to date. † Candidate gene for 9p deletion, XY sex reversal. BPES, blepharophimosis-ptosis-epicanthus inversus syndrome; GOF, gain-of-function mutation; LOF, loss-of-function mutation, ND, not determined; WAGR, Wilms' tumor-aniridia-genitourinary malformations-mental retardation.

mesonephric tubules appear to form normally, but tubules are absent from caudal regions, suggesting that mesonephric tubules are a heterogeneous population that form by at least two different mechanisms (201). During gonad development, *Wt1* is expressed in the coelomic epithelial cell layers and in the developing Sertoli cells in males and granulosa cells in females. Although the gonadal primordium can be observed in 11 dpc *Wt1*-mutant embryos, it degenerates thereafter by increased apoptosis (134).

Wt1 encodes a nuclear zinc-finger protein that can function as a transcriptional activator as well as a repressor, depending on the cell type and promoter context. It is expressed as a protein family due to RNA editing, alternative usage of translation start sites, and alternative splicing. Of particular interest is an alternative splice site that results in the insertion or exclusion of three amino acids (KTS) between zinc fingers three and four. The resulting isoforms play different roles in gonad development, which became obvious from the

generation of mouse strains in which the ability to produce one or the other specific isoform had been compromised (85). Mice of both sexes lacking the -KTS isoform had gonads that were markedly reduced in size and poorly differentiated, suggesting that WT1(-KTS) is required for the survival and differentiation of gonadal cells, probably through its function as a transcriptional activator. To date, a number of genes, including *Sf1*, have been shown to be activated by WT1(-KTS) in vitro, and experiments using transgenic mice strongly support the possibility that *Sf1* is a genuine WT1 target (244).

2. Steroidogenic factor 1

Sf1 is expressed in the developing urogenital ridge, hypothalamus, and the anterior pituitary gland, indicating the essential role in the development of the hypothalamic-pituitary-gonadal axis. After sexual differentiation, SF1 can be detected in steroidogenic (Leydig) and nonsteroidogenic (Sertoli) cells in the testis. Mice lacking a functional *Sf1* gene show complete failure of adrenal and gonadal development, obesity, and abnormalities of the ventromedial pituitary and hypothalamus gonadotropes (143, 200, 214). The gonads of *Sf1*-mutant embryos do not develop beyond the early indifferent stage with the result that XY animals show sex reversal in that the Müllerian ducts develop into uteri, oviducts, and upper vagina.

3. Lhx9

Lhx9 belongs to the LIM homeobox gene family, which is characterized by the presence of two NH_2-terminal LIM domains, predominantly involved in protein-protein interactions, followed by a DNA-binding homeobox domain (reviewed in Ref. 91). Intriguingly, the gonadal phenotype of $Lhx9^{-/-}$ mice is very similar to that of $Sf1^{-/-}$ and $Wt1^{-/-}$ mice (143). Indeed, biochemical analysis revealed that LHX9 can bind directly to the *Sf1* promoter and has an additive effect to the WT1-induced activation in vitro (244). These results represent a starting point for the assembly of a regulatory network of gene regulation during the bipotential stage of gonad development (Fig. 11).

4. Emx2

The homeobox gene *Emx2*, a mouse homolog of the *Drosophila* head gap gene empty spiracles (*ems*), is expressed in the developing dorsal telencephalon and in the epithelial parts of the urogenital system (254). In $Emx2^{-/-}$ mutants, the migration of the PGCs occurs normally, but the thickening of the coelomic epithelium, which marks the first stage of the gonadal development, is not prominent, and the Müllerian duct never forms. These mutants lack gonads and genital tracts completely (158). Nothing is yet known about the regulation and possible target genes of *Emx2* in the development of the early gonad.

5. M33

M33 is the murine counterpart of the *Polycomb* gene in *Drosophila*. It has been suggested that members of the Polycomb group proteins maintain a repressed state of homeotic and other developmentally regulated genes by compacting the chromatin and thereby preventing the binding of transcriptional activators. Homozygous *M33*−/− mice show male-to-female sex reversal in most XY animals (121). In addition, XX animals displayed no or smaller ovaries than wild-type littermates. Because gonadal growth defects were obvious in both sexes, it has been suggested that M33 plays a role in early gonad development before the time of sex determination (121). Recently, M33 has been implicated in the regulation of *Sf1* expression in spleen and adrenal gland (120). It may play a similar role in gonad development, but its exact cellular roles or molecular functions have not been studied in detail.

B. Genes Involved in Duct Formation

Other genes, in addition to *Emx2*, have been implicated in the initial formation of male and female genital tracts. *Pax2* is expressed in condensing mesenchyme and epithelial derivatives during kidney tubulogenesis (65). Expression of *Pax2* within the mesonephros is limited to the Wolffian ducts and mesonephric tubules. In $Pax2^{-/-}$ mice, the Wolffian duct is normal, but, with the exception of the most anterior cluster, the tubules form only as vestigial nubs on the Wolffian duct (45). No defects in gonadal development have been described in either sex in $Pax2^{-/-}$ mice (231), showing that formation of the mesonephric tubules is not a requisite for gonadal development.

Several *Wnt* genes are expressed in or around the Wolffian and Müllerian ductal systems and play a role in their development. *Wnt7a* is expressed at the anterior end of the mesonephros at 11.5 dpc, then throughout the Müllerian duct by 12.5 dpc. In *Wnt7a*−/− mice, the Müllerian duct is poorly developed in females and fails to regress in males (183). *Wnt4*, which is involved in the formation of pretubular cell aggregates in kidney morphogenesis (220), is expressed in the mesenchyme surrounding the ducts, especially concentrated around the Müllerian ducts. In *Wnt4*−/− mice, the Müllerian duct is absent in both females and males (239). *Wnt5a* is also expressed as early as 10.5 dpc in the mesenchyme surrounding the Wolffian duct (57), but data from null mutants have not yet been reported.

Another Lim-homeobox gene, *Lim1* (or *Lhx1*), plays a role in both male and female duct development. Mice homozygous for a null mutation in this gene lack all derivatives of Müllerian and Wolffian duct, i.e., female mice do not have oviducts, uteri, and upper vagina, and in male mice the rete testis, epididymis, vas deferens, and seminal vesicle are absent. *Lim1* seems to be required cell-autonomously in the developing epithelium of the ducts (128, 129).

C. *Sry:* The Molecular Switch

Up to this stage, the processes and factors that play a role in sexual development are the same in both males and females. This situation changes rapidly when, at around 10.5 dpc in mice, *Sry* starts to be expressed from the Y chromosome. As mentioned previously, *Sry* is necessary and sufficient for initiating testis determination and subsequent development of male sexual characteristics (at least in most mammals; some exceptions will be discussed below). If *Sry* is not present, or its function is impaired, an ovary will form. We first describe molecular mechanisms and pathways involved in testis differentiation before summarizing what is known about ovary development.

1. *Structure and function of SRY*

Sry encodes a member of a large family of nuclear proteins characterized by a DNA-binding domain, known as high mobility group (HMG) box (Fig. 12), according to its identification in a high mobility class of non-histone proteins that associate with DNA. There are two main classes of HMG proteins. Members of the first, such as HMG-1 and HMG-2, bind to DNA in a sequence-independent manner. In contrast, proteins that belong to the second class, which includes SRY, bind DNA sequence specifically. SRY binding to the minor groove of the DNA induces a sharp bend of 60–85°. Biochemical analysis of SRY protein expressed in human XY sex-reversed patients revealed that DNA binding and bending are integral parts of SRY function (88, 104, 190, 208). Almost all mutations found in SRY that cause XY sex reversal reside within the HMG box, also suggesting that other regions of the protein play only a minor role, if any at all (Fig. 12). This idea

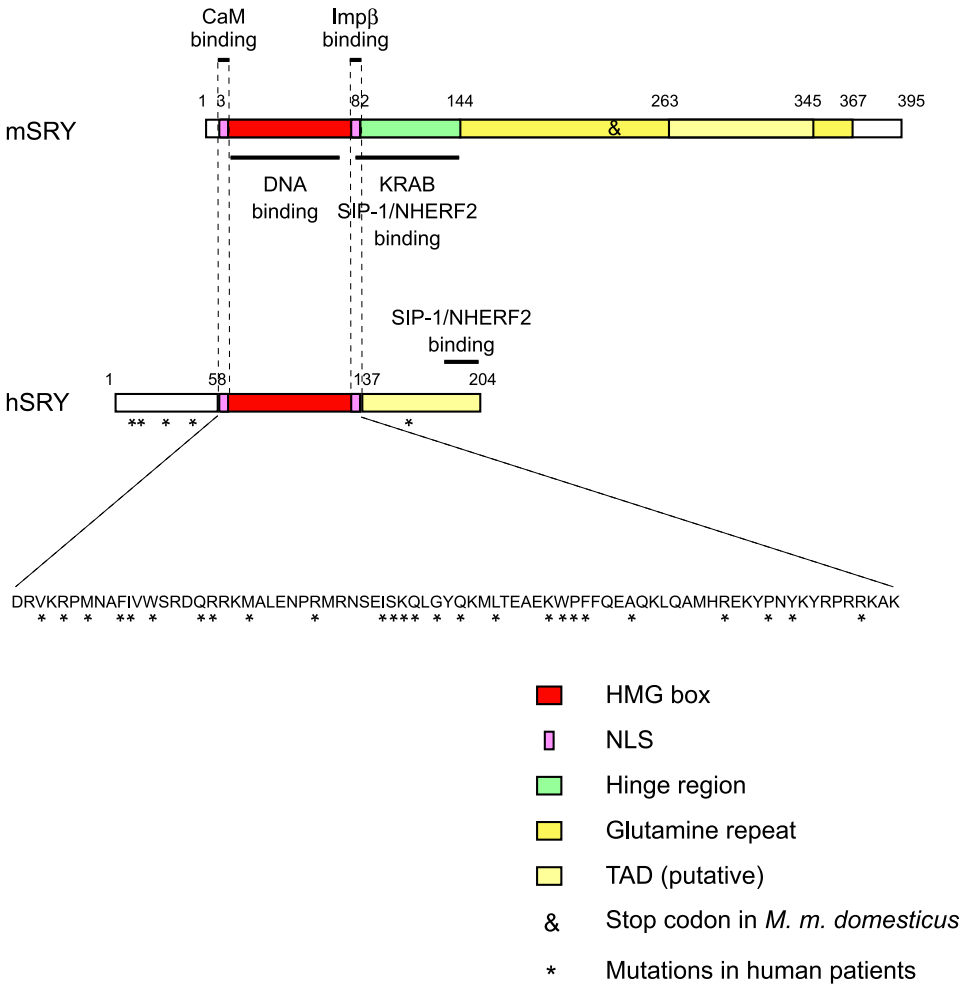

FIG. 12. Structure of mouse and human SRY protein. The HMG DNA-binding domain is shown in red and the large glutamine-rich domain of the mouse SRY COOH terminus is in dark yellow. Nuclear translocation is mediated by one NLS (pink) at either end of the HMG domain. The NH_2-terminal NLS is recognized and bound by calmodulin (CaM), whereas the COOH-terminal acts via importin β. For both mouse and human SRY, a putative transactivation domain (TAD, light yellow) has been described. The hinge or bridge region (green) interacts with mouse SRY-interacting protein 1 (SIP-1/NHERF2) and the KRAB-only protein, whereas human SRY interacts with SIP-1/NHERF2 via its COOH terminus. Sex-reversing mutations in human SRY (marked by asterisks) leading to gonadal dysgenesis or hermaphroditism are mainly found in the HMG domain.

is supported by the fact that non-HMG sequences are very poorly conserved between different species. These regions evidently have evolved rapidly, suggesting that no sequence restraints exist (119, 181, 235, 243).

How does SRY function? There are reports that SRY can act as a transcriptional activator (50, 68), but also as a repressor (63, 148). Consistent with a role as an activator, a *trans*-activation domain has been described for human and mouse SRY (67, 68). Removal of this domain impairs the ability of SRY to direct male development in transgenic mice (27). However, in mice, this domain exists only in certain subspecies, such as *Mus musculus molossinus*, but not in others, such as *Mus musculus domesticus* (67), and it is possible that the transgenic mouse data might reflect instability of a truncated SRY protein. It remains possible that in vivo the HMG box might be the only functionally important domain required to bind and bend DNA and thereby allow SRY to act as an architectural factor, and also required for interacting with other proteins (246). That scenario would leave the flanking regions with only a secondary role such as stabilization of the mRNA transcript and/or protein.

Interestingly, Oh et al. (173) reported recently the interaction of SRY with a novel protein containing only a Krüppel-associated box (KRAB) domain (173). This protein interacts with the hinge or bridge region, just COOH terminal of the HMG box of mouse SRY (Fig. 12). This region is the only part outside the HMG box that shows reasonable homology between different species and, when mutated, can lead to sex reversal in humans (211, 212). Furthermore, this KRAB-only protein interacts with a corepressor complex leading to gene silencing, which would support the long-standing hypothesis that SRY acts as a repressor (148). The same mouse SRY bridge region was also found to interact with SIP-1/NHERF2 (SRY interacting protein 1), which was identified for its interaction with human SRY (191, 228). But, like with KRAB-O, there is no in vivo evidence that this interaction occurs or plays any role during sex determination. Without the identification of a direct target of SRY, it will be difficult to assess the significance of these interactions in vivo.

2. Target genes of SRY

Although *Sry* was discovered 15 years ago, no in vivo target gene has been identified. It remains controversial whether *Sry* has multiple target genes or just one that carries out all functions necessary for initiating male sex development. The identification of an SRY target gene(s) remains one of the greatest challenges in the field.

Arguably the best candidate to date is *Sox9*, which starts to be expressed immediately after *Sry* and in a very similar spatial pattern. However, evidence that supports and contradicts this hypothesis has emerged (see below), and a final proof either way has not been forthcoming. Transgenic mice that express *Sox9* under the control of the *Wt1* promoter (241), and a mutant strain of mice, Odd Sex, in which Sox9 fails to be normally downregulated in XX development (192) both show XX sex reversal, suggesting that *Sox9* is the only target of SRY and is able to fulfill all its functions. However, another possible explanation would be that SOX9 is able to functionally replace SRY in these experiments because the expression of the transgene starts earlier than the expression of endogenous *Sox9* (42). This hypothesis was supported by findings that swapping the HMG box of *Sox9* into *Sry* and expressing it under the control of the *Sry* promoter also resulted in XX sex reversal (18). Clearly, it will not be possible to resolve this issue until target genes for both transcription factors are identified.

The only other SRY target gene that has been suggested so far is Wilms' tumor suppressor 1 (*Wt1*). However, evidence for this possibility came from overexpressing *Sry* in a mouse ES cell line (232), an artificial system in which the upregulation of *Sox9* after *Sry* expression could not be reproduced, suggesting that these data may not represent the in vivo situation.

In addition to the direct transcriptional regulation, SRY has been implicated to play a role in splicing and thereby posttranscriptional control of gene expression (174), but again only in vitro data exist and the significance in vivo and genes that might be regulated have not yet been determined.

3. Regulation of Sry expression

Sry expression in mouse gonadogenesis is tightly regulated and follows a curious and reproducible wave-like pattern. In situ hybridization and immunofluorescence revealed that *Sry* mRNA and protein expression starts at 10.5 dpc in the center of XY genital ridges, encompasses the whole length of the gonad, and reaches a peak at 11.5 dpc, and then begins to recede from anterior to posterior at 12 dpc, with the last positive cells detectable around 12.5 dpc at the caudal pole (35, 224, 245). Experiments in mice that possess a so-called "weak" *Sry* allele, a phenomenon known as B6-Y^{pos} sex reversal (70), showed that the correct timing of *Sry* expression is crucial for its function. In these mice, *Sry* expression starts too late and results in ovotestis formation when expressed from the Y chromosome of the *Mus musculus poschiavinus* mouse strain on a C57BL/6J background (34). Not only the timing, but also the expression levels of *Sry* are important for its function. Using the same experimental system, Nagamine et al. (165) showed that *Sry* expression level has to reach a certain threshold to induce testis development (165).

What factors regulate *Sry* expression and how? The first ideas came from three recently described null

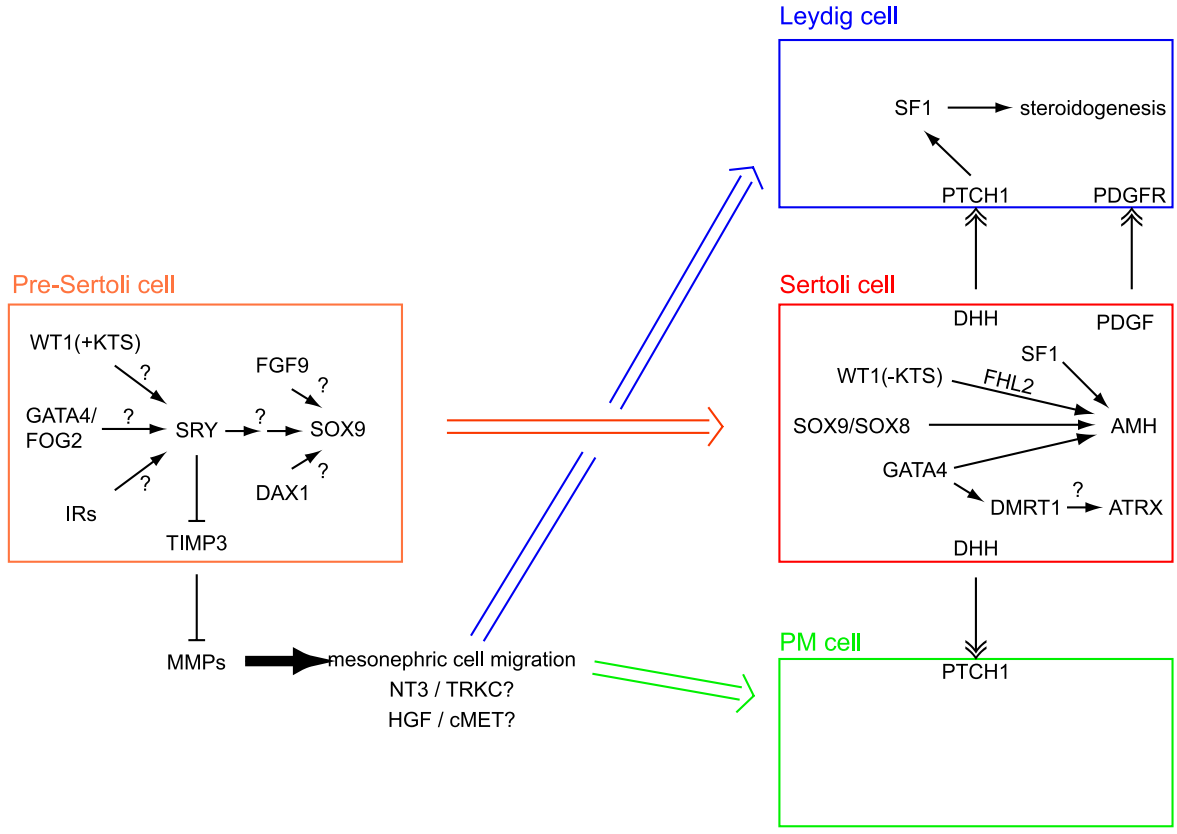

FIG. 13. Postulated interaction of molecular players involved in early testicular development. See text for details. Double-headed arrows, binding to a receptor; colored arrows (blue, red, green), differentiation of precursor cells into testis-specific cell types; black, bold arrow, gene important for cellular process.

mutation mouse models, WT1 (+KTS), GATA4/FOG2, and the insulin receptor family (Fig. 13). In all three cases, *Sry* expression levels were reduced to ~25% of wild-type levels, resulting in XY sex reversal (85, 170, 227). All three factors could be directly or indirectly involved in the regulation of *Sry* transcription, but it might also be possible that there are simply fewer cells that express *Sry* in these situations. Assuming the latter is not the case, at least the regulation by the insulin receptor family must be indirect, involving intracellular signaling pathways. Also, given the fact that on all gonadal promoters investigated in vitro so far FOG2 acts as a corepressor rather than as an activator (197), activation of *Sry* transcription is more likely to be indirect by the GATA4/FOG2 complex via the action of another factor. For WT1(+KTS), the situation is slightly more complicated. It has been shown that WT1(+KTS) binds preferentially to RNA, and not to DNA (47, 123), so it may be that the +KTS isoform functions at a posttranscriptional level by stabilizing the *Sry* mRNA. In addition to these examples with mouse mutant data, other possible regulators are all genes that are expressed in the genital ridge before *Sry* expression and whose mutation leads to gonadal agenesis before *Sry* is expressed, such as *Sf1* and *Emx2* (143, 158). In support of this possibility, de Santa Barbara et al. (62) showed that SF1 upregulated a human *SRY* reporter gene up to 2.5-fold using the human teratocarcinoma cell line NTERA2.

The above-mentioned B6-Y^{pos} sex reversal phenomenon indicates that SRY has to function within a defined window of time. What is not known is its effects or its significance after this critical time period. *Sry* expression in mice is extinguished by 12.5 dpc but persists in humans, sheep, and pigs (56, 86, 182, 185), raising several questions. Is the downregulation of *Sry* expression in mice an active process or is it due to the disappearance of an activating signal? Has SRY in humans and other species gained an additional function after the time of sex determination or is there no functional relevance of the later expression so that it was lost in mice?

Some clues are provided by the analysis of two transgenic mouse models, one in which *Sry* is ectopically expressed and not downregulated at the correct time, and another one in which the GFP-reporter gene is expressed under the control of the *Sry* promoter (4, 125, 209). These mouse models showed that prolonged *Sry* expression in mice has no obvious consequence for

the development of the gonads, supporting the hypothesis that it has no function at these stages of development and has disappeared in mice. Furthermore, these experiments implicated SOX9 as being responsible for the downregulation of *Sry* expression in mice; transgenic mice in which the levels of *Sry* expression are not sufficiently high to induce *Sox9* expression, as well as *Sox9* null mutants, do not turn off *Sry* (10, 48, 209). Similarly, a transgene consisting of a GFP-reporter gene driven by the *Sry* promoter shows the correct spatial and temporal upregulation observed for endogenous *Sry*, but its expression is maintained in the XX gonad where *Sox9* is not expressed (4).

4. Mammals lacking Sry: no rule without exception

Given the fact that *Sry* is regarded as the pivotal factor necessary and sufficient for mammalian male sex determination, it is surprising that there are mammals that determine male sex without the *Sry* gene (114). Examples of these exceptional animals are two species of the mole vole (*Ellobius lutescens* and *E. tancrei*) and two species of the Japanese spinous country rat (*Tokudaia osimensis osimensis* and *T. osimensis spp.*; Refs. 77, 221). In these species, males are fertile with fully functional testes and unambiguous genitalia, but do not have an apparent Y chromosome or the *Sry* gene in their genome. Even more surprisingly, other species of *Ellobius* and *Tokudaia* seem to have recognizable, normal Y chromosomes and express *Sry*. This phenomenon raises several interesting questions. Does it mean that *Sry* was lost during evolution, or did these species never have the *Sry* gene? Comparative studies revealed that the Y chromosome is progressively degrading and appears to have already lost most of its original 1,500 or so genes (80). It therefore seems likely that in the unusual *Ellobius* and *Tokudaia* species, *Sry* as a trigger for male determination was replaced by another gene, raising the possibility that *Sry* and subsequently the Y chromosome might become dispensable and replaced by another sex-determining gene in other species in the evolutionary future. Five genes (*Sox9*, *Sf1*, *Dmrt1*, *Pisrt1*, and *Foxl2*) have been excluded to function in this role in the unusual *Ellobius* and *Tokudaia* species (11, 12, 113), leaving several other genes that have been shown to play a role during testis differentiation (see below) as candidates.

D. Downstream Events in Testis Determination and Differentiation

It has been suggested that all mammals use a common downstream pathway of male sex determination, irrespective of whether *Sry* or some other gene constitutes the trigger mechanism. Whether this is true or not remains to be determined, as does the nature of the proposed common pathway. In the following sections we introduce genes that are known to play a downstream role and try to build a network of gene regulation and cell-cell interactions underlying testis differentiation (Fig. 13). Information about these and additional genes can be found in Table 1.

1. Sox9

As mentioned above, a good candidate as a genuine target of SRY is *Sox9*, which, like SRY, is a member of the SOX family of transcription factors. *Sox9* is expressed first at low levels in the indifferent gonad of both sexes, but becomes dramatically upregulated in the Sertoli cells immediately after the onset of *Sry* in a similar wave. However, in contrast to *Sry*, *Sox9* expression is maintained in the Sertoli cell until after birth. In addition, it is expressed at all sites of chondrogenesis (249, 260). Accordingly, heterozygous defects in human SOX9 have been associated with the bone dysmorphology syndrome campomelic dysplasia (CD). Interestingly, a large proportion of XY CD patients show sex reversal (76, 230, 242), suggesting that *Sox9* is necessary for male development. This theory was confirmed by the generation of conditional knockout mice that lack *Sox9* function in the developing gonads; gonads of these XY embryos develop as ovaries (10, 48). As discussed above, *Sox9* is not only necessary but also sufficient for male sexual development, because XX mice overexpressing *Sox9* develop as males (21, 241), suggesting that *Sox9* is the only important target of *Sry*.

This gonadal expression and function of *Sox9* is not only restricted to mammals, having also been described in chicken (162), which have a ZZ/ZW sex determination system, and in species with temperature-dependent sex determination such as the red-eared slider turtle *Trachemys scripta* (219). Even in *Drosophila*, the *Sox9* homolog *Sox100B* plays a male-specific role in the developing testis (58), placing *Sox9* in a central and conserved position in the process of sex determination.

Despite this pivotal role in male sexual differentiation, the regulation of *Sox9* expression within the developing gonads remains shrouded in mystery. It is often assumed to be directly activated by SRY, but the analysis of translocations affecting *SOX9* in CD patients suggested that regulatory regions upstream of this gene span over 1 Mb (188, 203, 248), making it difficult to prove a direct binding of SRY, or anything else for that matter. Comparative studies have revealed a number of putative regulatory motifs upstream of *SOX9* in humans, mice, and pufferfish (8). The regulatory potential of these motifs was tested using *lacZ* reporter transgenesis, and although they were identified as tissue-specific enhancers, none drove the expression in the testis (7).

Recent experiments suggest that the activity of SOX9 may also be subject to additional regulation at the level of subcellular localization (144, 145). SOX9 protein seems to be retained in the cytoplasm in the genital ridge of both sexes prior to 11.5 dpc, possibly via interaction with microtubules. After *Sry* expression in the XY gonad, it becomes phosphorylated, translocated into the nucleus where it regulates gene expression, and possibly its own upregulation, whereas in XX gonads SOX9 remains cytoplasmic and its expression ceases.

A further level of complexity arises from the observation that under several, seemingly unrelated circumstances *Sox9* transcription is activated in the absence of *Sry*. Mice mutant for both estrogen receptor α and β, or mutant for *FoxL2*, display XX sex reversal postnatally with *Sox9* expression in granulosa cells and subsequently their transdifferentiation into Sertoli cells (69, 175). Also, *Sox9* expression can be induced in ovaries by culturing them between a mesonephros and a testis or implanting it into a kidney capsule (162, 229). Moreover, *Sox9* is expressed in a male-specific manner in the developing gonads of many nonmammalian species that do not possess *Sry* at all. In all these cases SRY cannot be the transcriptional activator, and other ways of activating *Sox9* expression must exist.

Interestingly, XY mice that have a *Dax1* null allele and a Y allele from *Mus domesticus poschiavinus*, or that are mutant for the signaling molecule fibroblast growth factor 9 (FGF9), show reduced *Sox9* levels resulting in sex reversal, even though *Sry* levels are similar to wild-type XY mice (153, 154, 206). These observations suggest that DAX1 and FGF9 play a role in upregulating *Sox9* expression in pathways parallel to *Sry*. Whether these two molecules are also involved in the cases described above remains to be elucidated.

Sox9 represents a classical transcription factor with two defined *trans*-activation domains and two independent nuclear localization signals. However, to date, only limited information is available on what genes are activated by SOX9 that play a role in testis determination. In an elegant study, Arango et al. (5) showed that SOX9 together with SF1 is responsible for the regulation of anti-Müllerian hormone (*Amh*) gene expression. Whereas SOX9 plays an essential role in the initiation of *Amh* transcription, SF1 appears to have a modulatory effect on the level of transcription (5). Based mainly on in vitro studies, several other factors have been implicated to play additional roles in the regulation of *Amh* expression, suggesting a multiprotein complex consisting of SOX9, SF1, GATA4, WT1(-KTS), FHL2, a four-and-half LIM-domain protein, and probably yet unknown factors to synergistically activate the *Amh* promoter (61, 66, 95, 96, 233).

Apart from *Amh*, only two other genes have been described so far as being regulated by SOX9 that are involved in testis development. First, in vitro experiments suggested that SOX9 is responsible for the maintenance, but not initiation, of *Sf1* expression in Sertoli cells (213). Second, similar to *Amh*, the expression of *Vanin-1* seems to be dependent on SOX9 and SF1 (247). *Vanin-1* encodes for a pantetheinase, an enzyme that produces vitamin B_5 and the antioxidant cysteamine, which might serve to protect the PGCs from oxidative stress. Taken together, there is certainly a need to identify other SOX9 targets that are relevant to sex determination.

What other genes might be upregulated by SOX9? In addition to its role in sex determination, *Sox9* is expressed in chondrocytes and activates the expression of type II collagen and several other extracellular matrix proteins such as aggrecan and CD-RAP (16, 137, 171, 210, 250). It is therefore conceivable that genes encoding extracellular matrix proteins that are part of the basement membrane of the testis cords may be regulated by SOX9 in Sertoli cells.

2. *Sox8*

Another SOX protein shown to be expressed in a similar temporal and spatial pattern to SOX9 in the developing gonad is SOX8. Its expression is upregulated in Sertoli cells ~12 h after *Sox9*, but precedes the expression of *Amh* (202). On the basis of sequence similarities, *Sox8* belongs with its closest homologs, *Sox9* and *Sox10*, to the E group of *Sox* genes. SOX8-deficient mice have only minor defects such as a reduction in the overall body weight (218), probably because *Sox8* is mainly coexpressed with one or both of the other subgroup E members that are likely to compensate for its absence. In support of this postulated redundancy, SOX8, like SOX9, can bind to the *Amh* promoter in a sequence-specific manner and interact with SF1 to synergistically activate *Amh* expression in vitro (202). The redundant function of *Sox8* and *Sox9* in testis differentiation was recently confirmed in vivo by double knockout analyses, which showed that *Sox8* is able to reinforce *Sox9* function in testis differentiation in mice (48). It has been suggested that the apparently subsidiary role of *Sox8* in sex determination is a relic of its shared ancestry with *Sox9* and that *Sox8* may lose its function during sex determination as further evolution proceeds (131).

3. *Fgf9*

Also implicated in Sertoli cell specification is the secreted signaling molecule FGF9. *Fgf9* is broadly expressed in the mouse embryo (52) with a sex-specific pattern in the developing gonad. It can be detected from 11.5 dpc in both sexes (206). Later, it is restricted to the testis cords of the XY gonad, but not expressed in the XX gonad or the mesonephros of both sexes. Mice homozygous for a null mutation of *Fgf9* show XY sex reversal on

some genetic backgrounds but not others, whereas XX gonads develop normally (51, 206). The XY sex reversal is most likely due to a reduced proliferation rate, possibly combined with impaired differentiation of pre-Sertoli cells (204), such that a threshold number necessary for directing the differentiation of other cells in the gonad towards complete testis differentiation is not reached (36).

4. Dmrt1

A gene family involved in sex differentiation in organisms as phylogenetically disparate as *Caenorhabditis elegans*, *Drosophila*, fish, mammals, and coral, is the DM gene family (157, 194). In 1998, a human gene was discovered with homology to the *Drosophila* sex regulatory gene *doublesex* and the *C. elegans* sex regulator *mab-3* (195). All three genes encode proteins related by a common DNA-binding domain, dubbed the DM domain. More intriguingly, the human gene *DMRT1* maps to a region of chromosome 9p that, when deleted, is associated with XY gonadal dysgenesis (240). Subsequently, *Dmrt1* was discovered in chickens, mapping to the Z sex chromosome (167), thus adding to evidence that it might be involved in sex determination. Moreover, in humans, mice, chickens, alligators, and turtles, *Dmrt1* expression was detected in the developing gonads, and only the gonads (59, 124, 160, 194, 216), at higher levels in testes compared with ovaries, usually in the late sex-determining or early testis-differentiation period.

The most direct evidence for an important role for *Dmrt1* has come from studies in the model fish medaka (*Oryzias latipes*), where a related gene *DMY* (or *Dmrt1bY*) has been definitively identified as the primary, Y-linked sex-determining gene (147, 166). However, the null mutant mice showed only a relatively mild phenotype in postnatal testis differentiation (194), suggesting that either it is not important for primary sex determination or other factors are able to compensate for its loss. Indeed, Kim et al. (126) described recently three other DM proteins that are expressed in the developing gonad: *Dmrt3*, *-5*, and *-7*. Of these, *Dmrt3* might be of interest because it is expressed in the same temporal and spatial pattern as *Dmrt1*. A definitive demonstration of the importance of *Dmrt1* for sex determination in mammals awaits the production of multiple DM-gene knockout mice and/or gain-of-function transgenic mice.

5. Dax1

DAX1, encoded by the X-chromosomal gene *Dax1* (or *Nr0b1*), is an atypical member of the nuclear receptor superfamily. Its NH_2 terminus contains, in place of the otherwise highly conserved DNA-binding domain, a repeated peptide sequence LXXLL that mediates protein-protein interaction (222, 257, 259), whereas the COOH terminus harbors a *trans*-repression domain (101, 258). The main model of the molecular mode of action of DAX1 is thought to be the interaction with other nuclear receptors such as SF1 (135, 164, 222), the estrogen receptors ERα and ERβ (259), the androgen receptor, and the progesterone receptor (3, 93), recruiting corepressors to the appropriate transcriptional complex and thereby inhibiting nuclear receptor-mediated transcriptional activation. Nevertheless, this does not exclude the possibility that DAX1 can directly regulate gene expression by binding to DNA or RNA (for review, see Ref. 142).

Dax1 is expressed in the developing adrenal gland, the gonads, the hypothalamus, and the pituitary (86, 98, 99). During testicular development, *Dax1* is expressed in somatic cells before *Sry* is expressed, with a strong upregulation in Sertoli cells by 12.5 dpc, which declines thereafter. However, there is a second increase in expression, this time in interstitial cells, between 13.5 and 17.5 dpc. In contrast, *Dax1* continues to be expressed in the developing ovary until at least 14.5 dpc (99).

Based on duplications in humans that led to XY sex reversal, *Dax1* was thought to be an ovarian-determining or anti-testis gene (9, 223). Therefore, it came as a surprise that loss of function in female mice had no reproductive consequences, but testicular development in XY animals was impaired (26, 153, 154, 255). The severity of the phenotype is dependent on the genetic background and ranges from reduced testicular size and impaired germinal epithelium development (255) to complete sex reversal (26, 154). The molecular mechanism underlying this testis-promoting versus anti-testis function of *Dax1* is not clear. Dosage sensitivity during gonadal differentiation appears to play an important role in humans and mice, which makes it feasible that a fine balance of *Dax1* levels at the right time could tip the balance in either of the two ways (142).

6. Cross-talk between Sertoli cells and other cell types

Evidence reviewed in section III indicates that Sertoli cells are the organizing centers of the developing testis. To fulfill this function they have to communicate with and instruct the cells around them by expressing secreted factors that bind to specific receptors on the receiving cells (29). Some of these have been identified and are discussed in the following paragraphs (Fig. 13 and Table 1).

By far the most studied molecule secreted by Sertoli cells is anti-Müllerian hormone (AMH), also called Müllerian-inhibiting substance (MIS). AMH belongs to the transforming growth factor-β superfamily, which signals by binding and assembling two related serine/threonine kinase receptors. The MIS type II receptor (MISIIR) is expressed in the mesenchyme surrounding the Müllerian duct in XY and XX animals, and in Sertoli and granulosa

cells in embryonic and adult testes and ovaries, respectively. AMH is responsible for Müllerian duct regression in males by triggering a BMP-like signaling pathway through MISIIR and probably the type I receptor ALK2. However, this is likely to be an indirect effect because these receptors are expressed in the mesenchymal cells surrounding the Müllerian duct, but apoptosis is induced in cells of the Müllerian duct epithelium. Recently, MMP2, a member of the extracellular matrix metalloproteinase family, has been identified as a candidate target of AMH/MISIIR signaling. It seems to function as a paracrine death factor causing apoptosis in the epithelial cells (198). Interestingly, male mice carrying null alleles for *Amh* retain their Müllerian ducts but develop normal testes. Therefore, *Amh* does not seem to have an essential role during male gonad development (15).

Another cell signaling molecule secreted by Sertoli cells, desert hedgehog (DHH), seems to play a role in regulating Leydig and peritubular myoid cell function. Among the three hedgehog genes described in mammals, *Desert*, *Indian*, and *Sonic hedgehog*, only *Dhh* is expressed in the developing gonad. Its expression starts at 11.5 dpc in somatic cells of XY mouse embryos and continues thereafter in Sertoli cells. In contrast, no expression can be observed at any stage in the developing ovary (23, 251). DHH binds to its receptor Patched 1 (PTCH1), which is expressed shortly after, and under the positive control of, *Dhh* on Leydig and peritubular myoid cells (49, 251). Null mutation of *Dhh* in mice resulted in disrupted formation of testis cords due to abnormal peritubular tissue (49, 189). In addition, *Dhh* seems to be necessary for Leydig cell differentiation by upregulating *Sf1* in these cells (253).

In addition to DHH, PDGF is an important component for the differentiation of Leydig and peritubular myoid cells. PDGF has been described as a major mitogen for connective tissue cells and certain other cell types. It is a homo- or heterodimeric molecule consisting of A-, B-, C-, and/or D-polypeptide chains. These molecules exert their cellular effects by binding to α- and β-protein tyrosine kinase receptors. *Pdgfr*α is expressed at 11.5 dpc at low levels in the mesonephros and at high levels in the coelomic epithelium and at the gonad-mesonephros border in both sexes, as well as in the XY gonad itself. XY mice deficient for PDGFRα have a severe reduction in mesonephric cell migration, and therefore disrupted sex cord formation and organization of the vasculature and impaired Leydig cell differentiation (31). Interestingly, the role of PDGFRα in mesonephric cell migration is dependent on its expression on gonadal cells and not the migrating cells, suggesting that it acts in an indirect way.

Among the secreted molecules still to be identified as important mediators of Sertoli cell signaling are those involved in inducing mesonephric cell migration, cell proliferation, and the mitotic arrest of germ cells around 12 dpc.

Recently a gene has emerged that is impossible to categorize specifically as "testis" or "ovary" gene (54). *Pod1* encodes a basic helix-loop-helix transcription factor involved in kidney, facial muscle, lung, and spleen development (140, 141, 193). XX as well as XY mice deficient for *Pod1* develop hypoplastic gonads and abnormal vascular development, resulting in feminized external genitalia. The molecular basis of this phenotype might be explained by the expanded *Sf1* expression and therefore expanded steroidogenic cell population observed in gonads of both males and females of these mutant strain (54).

E. Ovarian Development: Terra Incognita

The early development of the vertebrate ovary is poorly understood at the histological, cellular, and molecular levels, a rather amazing situation given the importance of this organ for proper female development and reproduction. A contributing factor to this situation is the genetically dominant role of the testis-determining pathway and the discovery of several key components of this pathway, including *Sry* and *Sox9*. Moreover, the prevailing view that ovarian development is the "default" state has commonly led to an incorrect assumption that no active genetic steps need to be taken to specify or create an ovary. This cannot really be the case, however, and several lines of evidence argue that ovarian development must involve the coordinated activity of a large number of genes. First, the ovary is composed of a number of specialized cell lineages arranged in an ordered configuration, and common sense would suggest that many genes are required to orchestrate the cell differentiation, migration, proliferation and programmed death, and the intercellular signaling events involved with morphogenesis of this complex organ. Second, numerous human disorders involve ovarian dysgenesis, implying that active control mechanisms must occur in early ovarian development. Third, studies of genetic sex reversal in mice indicate that there is a narrow time window during which *Sry* must act to induce testis development, implying that that this window is delimited by the time of action of competing, active ovarian determining factors.

We discuss here evidence bearing on the possible roles of three genes likely to be involved in early development of the ovary.

1. Wnt4

Results from a recently generated mouse model further refute the concept that ovarian development is a passive, default pathway. *Wnt4*−/− XX mice show partial sex reversal, suggesting that *Wnt4* acts to positively reg-

ulate ovary differentiation. In these animals the gonad has the appearance of a testis: round, unencapsulated, and associated with a fat body; the Müllerian ducts are missing and the Wolffian ducts further differentiate. However, the gonads do not form testis cords or express Sertoli cell-specific markers (239).

Expression of *Wnt4* is first observed in the mesonephric mesenchyme and coelomic epithelium from 9.5 dpc onwards. At 11 dpc, *Wnt4* is expressed in the mesenchyme of the indifferent gonad and the mesonephros of both sexes, but is downregulated in the male gonad ~12 h later. The expression persists in the mesonephroi as well as in the female gonad and the mesenchyme surrounding the Müllerian ducts (220, 239). *Wnt4* seems to have several roles in sexual differentiation. As mentioned previously, *Wnt4* is required during the indifferent stage for initial Müllerian duct morphogenesis in both sexes (239).

In addition, *Wnt4* inhibits the formation of the male-specific vascularization in the ovary and the separation of adrenal steroidogenic cells from the male and female gonadal primordium (89). However, in gain-of-function studies, male mice (which ectopically express *Wnt4*) still form a coelomic vessel, although the structure and the branching is abnormal (107). This suggests that either WNT4 is not the only factor responsible for repressing male-specific vascularization in the ovary, or testes express a factor that can overcome, at least in part, WNT4-mediated repression. *Wnt4* seems to exert these functions via the upregulation of *follistatin*, as suggested by expression and null mutation analysis (252). Both *Wnt4*- and *follistatin*-null ovaries develop the male-specific coelomic vessel, and a massive loss of germ cells by apoptosis at 16.5 dpc depletes the entire pool of oocytes.

2. Dax1

Another possible target gene of WNT4 signaling is *Dax1*, which is female specific and expressed from 12 dpc onwards. In vitro studies showed that *Dax1* is upregulated by *Wnt4* and that the WNT/β-catenin pathway mediates this activation (159). This hypothesis is supported by a human case of gonadal dysgenesis caused by *WNT4* duplication, in which the phenotype resembles that seen in patients with *DAX1* duplication (110). In humans, duplication of the region Xp21 that encompasses the *DAX1* gene causes 46,XY individuals to develop as females, a situation that led to the original hypothesis that *Dax1* might be the ovary-determining gene (163, 223, 257). However, as discussed, inactivation in mice does not impair ovarian development or other aspects of female sexual differentiation, but causes spermatogenic deficiencies and defects in early testis development (255). The current view is that *Dax1* has roles in both testicular and ovarian development, apparently involving different dosage requirements. Clearly this is a complex biological situation and one that will require some experimental ingenuity to unravel.

3. FoxL2

Despite their indubitable importance, neither *Dax1* nor *Wnt4* has proven to be the ovarian-determining factor. The only other candidate that has emerged for this role to date is *FoxL2*. FOXL2 is a member of the large family of forkhead/winged helix transcription factors, known to play important roles during vertebrate development (for review, see Ref. 122). *FoxL2* is expressed in a female-specific manner in the gonads from 12.5 dpc, and this expression pattern is conserved between different phyla (139, 207). It is expressed in mesenchymal pregranulosa cells and later in granulosa cells before its expression ceases postnatally (207). Furthermore, *FoxL2* plays a role in the autosomal XX sex reversal phenotype of the polled intersex syndrome in goats (176).

FOXL2 also has been implicated in a human congenital disease, blepharophimosis-ptosis-epicanthus inversus syndrome (BPES; Ref. 53), which is characterized by eyelid abnormalities and is often associated with premature ovarian failure, implying a functional role in ovarian development or maintenance. Studies of mice carrying a null mutation in *FoxL2* (175, 207, 236) revealed that this gene is essential for granulosa cell differentiation and as a result for ovary maintenance. Absence of functional granulosa cells evidently leads to premature initiation of folliculogenesis and subsequently ovarian failure, which provides a molecular mechanism for the ovarian phenotype in BPES (207). However, there is no BPES case with female-to-male sex reversal, and *Foxl2* null mutations in mice do not result in defects in early ovary formation (207, 236), excluding FOXL2 as the ovary-determining factor.

4. Towards a pathway of ovarian development

The lack of genes known to be expressed in the early ovary has precluded attempts to put together a pathway of gene regulation of early ovarian development. However, recent large-scale transcriptional analysis revealed a surprisingly large number of genes to be upregulated in the developing ovary from as early as 11.5 dpc (28, 33, 111, 169, 252), indicating that there is a robust, female-specific genetic program in place at far earlier stages than previously suspected. The number of genes is lower than in males, which probably means that the program is activated slightly later than the testis-specific program. It will be particularly important to identify genes that are specific for the different ovarian cell types that can be used as markers for further studies to provide an understanding of how early ovarian development proceeds. This in turn

should lead to elucidation of the regulatory pathways orchestrating early ovarian differentiation.

V. CONCLUSIONS

The mechanisms by which sex is determined throughout the animal kingdom show a surprisingly high degree of variability, considering that correct sex determination and differentiation is a prerequisite for reproduction and therefore imperative for the survival of all sexually reproducing species. In mammals, it has been known for 15 years that the Y-chromosomal gene *Sry* is necessary and sufficient for male sex differentiation, and many others genes have since been implicated in testis development. Expression screens such as microarray analyses have resulted in hundreds of candidate genes that show sex-specific expression patterns. However, it has been difficult to place these genes into a network of gene regulation and function. Even for *Sry*, it is still not known how its expression is regulated, what proteins might interact with it, and which genes it regulates. Corresponding studies of ovarian development are even less advanced.

Insight into the regulatory networks underpinning correct development of the gonads is a prerequisite for understanding the molecular mechanisms of human intersex disorders. It is estimated that 1 in 100 live births shows some sort of gonadal dysgenesis, ambiguous genitalia, genital malformation, or even sex reversal. Nevertheless, most of these disorders are still unexplained at the molecular level.

Because of its dimorphic nature, gonad development is a fascinating example of organogenesis, in which one common primordium has the potential to develop into either of two morphologically and functionally different organs, testis or ovary. This situation presents a unique opportunity to study molecular mechanisms and networks of gene regulation that result in the formation of functional organs, which most likely can be translated into other systems of organogenesis.

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