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Sexual Differentiation of Somatic and Germ Cell Lineages of the Mouse Gonad

by

Douglas B. Menke

B.S. Biology and Chemistry
Trinity University, 1994

SUBMITTED TO THE DEPARTMENT OF BIOLOGY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

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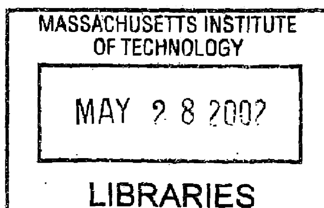
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Submitted to the Department of Biology on May 10, 2002
in Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy in Biology

Abstract

The process of gonadal sex differentiation in mammals remains poorly understood despite the identification of a few key genes such as *Sry* and *Sox9*. To further our understanding of gonad organogenesis, a PCR based subtractive hybridization was performed to identify mouse genes that demonstrate sexually dimorphic expression patterns during embryonic development of the testis and ovary. I successfully identified nineteen genes that are upregulated during testis differentiation and two that are upregulated during ovary development. These genes are expressed predominantly in testicular and ovarian somatic cell types and encode several different classes of proteins.

I analyzed the expression of these genes during the initial stages of gonadal sex differentiation and found that testis genes are upregulated in a center-to-pole pattern in which expression occurs in the central region of XY gonads and subsequently spreads to the anterior and posterior poles. In XX gonads I found that one ovarian gene, *follistatin*, is also expressed in a center-to-pole pattern, whereas a second ovarian gene, *Adamts19*, is expressed in an anterior-to-posterior wave. In addition, these ovarian genes are upregulated at the poles of XY gonads, suggesting that ovarian gene expression patterns are initiated at the poles of XY gonads before testis specific expression patterns are established.

I have also found that the sex differentiation and meiotic entry of female germ cells occurs in an anterior-to-posterior wave. The presence of this wave indicates that local environmental cues in the ovary may regulate meiotic entry. I identified this anterior-to-posterior wave of differentiation using the *Stra8* gene, which I show is a molecular marker of female sex differentiation. Through the generation of *Stra8* knockout mice I have further demonstrated that this gene is required for male and female meiosis. The absence of *Stra8* homologs in non-vertebrate species suggests that the study of this gene may provide unique insights into vertebrate meiosis.

Finally, I describe the characterization of *Tet14*, a testis specific X-linked gene. *Tet14* encodes a novel open reading frame that lacks homology to other known genes. I have generated a conditional knockout to determine the functional requirement for TET14 in embryonic and adult mouse testes.

Thesis Supervisor: David C. Page
Title: Professor of Biology, Howard Hughes Medical Institute

To my parents, who encouraged my interests
and helped me pursue them to the end

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My time in the Page lab has been extremely rewarding, in large part due to the people I've had the fortune to work with. David Page ignited my interest in germ cell biology, and his unflagging and spirited support helped propel my research forward. Bruce Lahn, my friend and graduate student mentor, was a tremendous source of advice and ideas during my early graduate years. More recently, Alex Bortvin and Jeremy Wang have provided me with many insightful discussions. Laura Brown has always been generous with her friendly advice, and Mary Goodheart's enthusiastic help with my mouse work has made all the difference. My friendships with fellow graduate students Charles Tilford, Richa Saxena, Julie Bradley, Aaron Schwartz, Jennifer Saionz, YanFeng Lim, Jesse Potash, Andy Baltus, and Julian Lange have been truly enjoyable. In particular, Charles Tilford's irrepressible nature and dry sense of humor kept life in the Page lab interesting. Finally, I want to thank Anna Lau for her unwavering support and encouragement that helped me through on those frustrating days.

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Chapter 1

Introduction

The mammalian testis and ovary share a common function: the generation of haploid germ cells. Yet the architecture of the testis and ovary, and the appearance of germ cells that they produce, is vastly different. Amazingly, the male and female gonads originate from a common precursor that is capable of differentiating into either a testis or an ovary. Our understanding of the process of gonadal sex differentiation is expanding, but is still far from complete. We have some knowledge of when certain cell types differentiate, where they originate from, and even some of the genes they express, but this knowledge is fragmentary in nature. Many of the genes involved in testis and ovary development remain to be identified, and of the genes that have been identified the functional analysis has often been complicated by requirements for these genes at multiple stages of gonad development.

The common origins of the testis and ovary

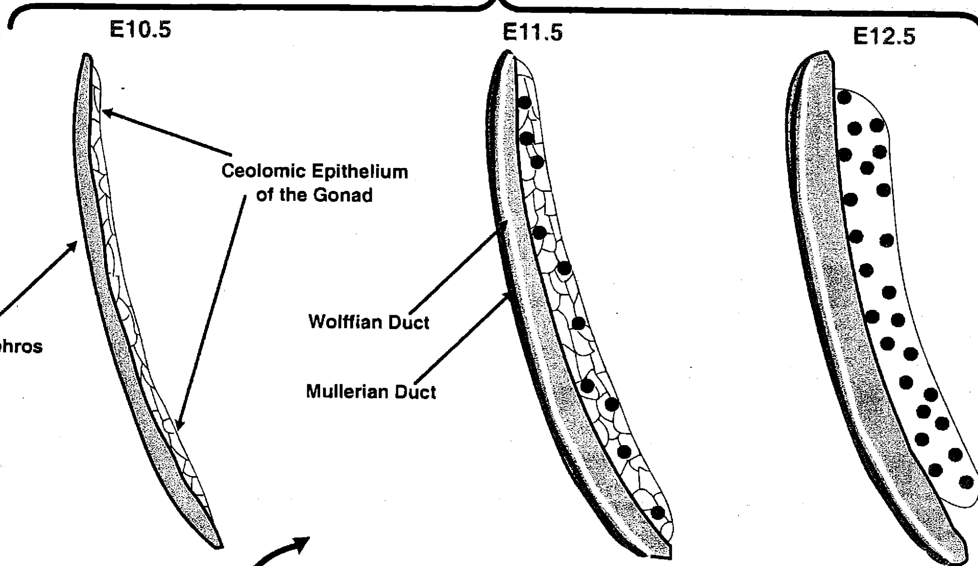
The mammalian testis and ovary develop from a primordial ridge of tissue during embryonic development that is common to XX and XY embryos. This tissue is often referred to as the "bipotential gonad" or as the "genital ridge." In the mouse embryo the rudimentary gonad is first identifiable at embryonic day 10 (E10), approximately midway through mouse gestation (McLaren, 2000). The gonad initially forms as an outgrowth of the mesonephros that, along with the pronephros and the metanephros, is derived from a region of mesodermal tissue called the nephrogenic cord (Byskov and Hoyer, 1994). The metanephros gives rise to the adult kidney, while the pronephros is vestigial in mammals. The mesonephros functions as an embryonic kidney in certain mammals, though not in the mouse. In addition to providing the gonadal point of origin, the mesonephros gives

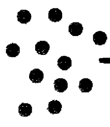
rise to the Mullerian and Wolffian ducts that ultimately differentiate into the female and male reproductive ducts respectively.

The bipotential gonad first forms as a thickening of the ceolomic epithelium on the ventral surface of the mesonephros (Fig. 1). The gonadal tissue is initially only a few cell layers deep, but by E11.5 the genital ridge has grown substantially in both XX and XY embryos (Capel, 2000). Throughout this early time period the germ cells, which arise extraembryonically, arrive at and enter into the genital ridges. The movement of the germ cells initially occurs passively as the tissue surrounding the germ cells involutes inward towards the genital ridges, though later the germ cells actively migrate into the gonads (Molyneaux et al., 2001). By E11.5 the majority of the germ cells have reached the genital ridges and are scattered throughout the gonad. The somatic cells and the germ cells are not organized in any obvious way at this time, and XX and XY gonads are still indistinguishable based on their appearance. However, between E11.5 and E12.5 XY, gonads undergo a dramatic reorganization in which a subset of the somatic cells enclose the germ cells and form the testicular cords (Fig. 1). The testicular cords, also known as the seminiferous tubules, are the most prominent feature of embryonic and adult testes. The cords contain the male germ cells as well as Sertoli cells, a population of somatic cells that support and nurture the development of the germ cells. A second somatic cell type, the peritubular myoid cell, forms a thin filamentous layer that surrounds the Sertoli cells and the germ cells. The third major somatic cell type of the testis is the Leydig cell. Leydig cells are steroid producing cells and are located in the interstitial spaces between the testicular cords. During these early developmental stages, XX gonads do not undergo a reorganization comparable to that which occurs in XY gonads. In fact, it is only late in

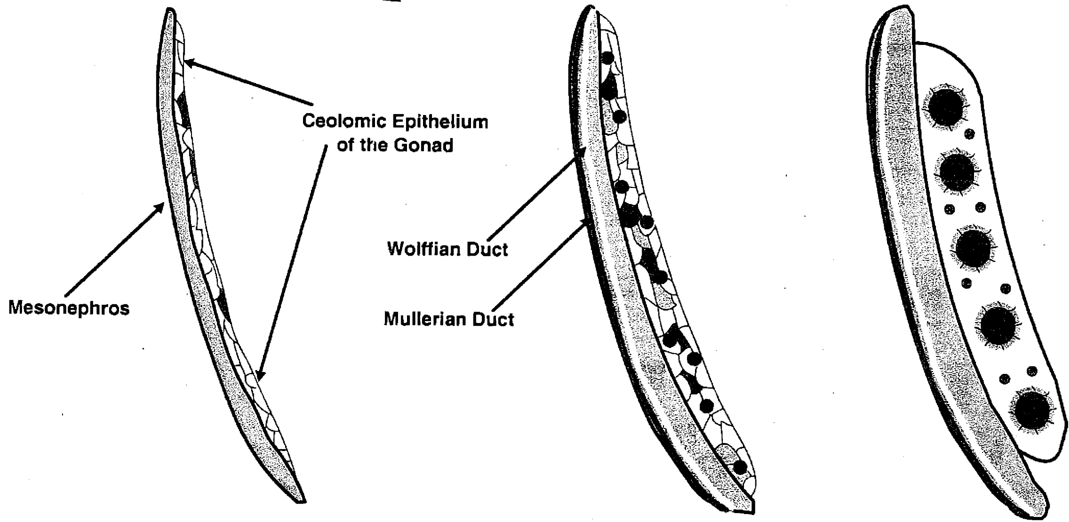
Figure 1. Early stages of gonad development and sex differentiation of the XX and XY genital ridge. The bipotential gonad arises from the mesonephros (blue crescent). (Top) The XX gonad exhibits significant growth during the early stages of gonad development, but somatic and germ cells remain disorganized. (Bottom) The growth observed in XY gonads after E11.5 is more extensive than that observed in XX gonads. This is due in part to a higher rate of proliferation of XY somatic cells, but is also a result of cells migrating into to the XY gonad from the mesonephros (blue cells, E11.5). Between E11.5 and E12.5 the XY gonad undergoes a dramatic reorganization as the testicular cords form. The Sertoli cells differentiate from precursor cells in the ceolomic epithelium of XY gonads (bottom left, red cells). The peritubular myoid cells originate from the mesonephros (blue cells).

XX Genital Ridge



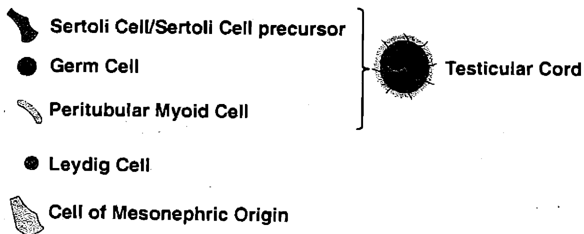


Germ Cells Migrate Into Gonads



E10.5 E11.5 E12.5

XY Genital Ridge



embryogenesis that ovarian granulosa cells, the female analog of the Sertoli cell, organize around the developing oocytes.

The growth that is observed in the bipotential gonad between E10 to E11.5 occurs through cell proliferation within the gonad and probably through the recruitment of additional cells from the mesonephros (Schmahl et al., 2000). After E11.5 there is clearly little or no migration of cells from the mesonephros into XX gonads. In contrast, there is significant migration into XY gonads until E16.5 (Martineau et al., 1997). The presence of this cell migration was elegantly demonstrated by Martineau and colleagues through a series of organ culture experiments in which E11.5 XY and XX gonads were dissected away from their adjoining mesonephroi and placed onto donor mesonephroi from mice that ubiquitously express β-galactosidase. After culturing these gonad-mesonephros complexes for two days, staining of the tissues for β-galactosidase activity identified cells that had migrated into the gonads from the donor mesonephroi. These experiments not only revealed that migration occurs into XY and not XX gonads after E11.5, but also demonstrated that the sex of the donor mesonephros does not affect the ability of XY gonads to induce cell migration.

The contribution of mesonephric cells appears to be critical for testicular development. Prior to the performance of the donor studies described above, it had been observed that E11.5 XY gonads grown in organ culture can undergo normal testis development and form testicular cords if the mesonephros is kept in contact with the gonad (Buehr et al., 1993). If E11.5 XY gonads are cultured in the absence of a mesonephros or if a thin membrane is placed in between the gonad and the mesonephros, then testicular cords will not develop. The use of marked donor mesonephroi revealed

that the cells that migrate from the mesonephros contribute significantly to the peritubular myoid cells that help to form the testicular cords (Martineau et al., 1997; Tilmann and Capel, 1999). This fact, in combination with the knowledge that ovaries are not known to contain myoid type cells, has lead to a model whereby the differentiating XY gonad recruits the precursors of the peritubular myoid cells from the mesonephros to help form the cords. No migrating cells were observed to contribute to the Sertoli cell lineage. However, cell labeling experiments using fluorescent dyes have demonstrated that Sertoli cell precursors originate from the ceolomic epithelium of the gonad prior to E11.5 (Karl and Capel, 1998). While it is possible that some Sertoli cell precursors migrate from the mesonephros, the cell migration experiments just described suggest that any contribution from the mesonephros would have to occur prior to E11.5.

The Sertoli cell as the testis organizer

Over the last two decades there has been a growing body of evidence that the Sertoli cell is responsible for recruiting and organizing the other testicular cell lineages of the testis. The first suggestive evidence for this was the observation that the Sertoli cells are the first somatic cell lineage to undergo obvious morphological differentiation during testis development (Magre and Jost, 1980). Much more compelling evidence eventually came from the analysis of XX↔XY chimeric mouse gonads (Burgoyne et al., 1988; Palmer and Burgoyne, 1991). When the contribution of XX and XY cells to different testis lineages in these chimeras was determined, it was found that the vast majority of Sertoli cells were XY, while XX and XY cells contributed equally to Leydig cells and peritubular myoid cells. These results provided the basis for a model of testis development in which the Sertoli cell precursors undergo cell autonomous differentiation

into Sertoli cells when a Y chromosome is present in the cell. It was further postulated that the granulosa cells of the ovary share a common precursor with the Sertoli cells, and that in the absence of a Y chromosome these precursors develop as female granulosa cells rather than male Sertoli cells. Since the differentiation of non-Sertoli testicular cell types can occur in the absence of a Y chromosome, this supported a model in which the differentiating Sertoli cells recruit additional testicular cell types.

When the testis determining factor of the Y chromosome, *Sry*, was first identified, it was found that *Sry* is expressed in the genital ridges of XY mouse embryos from E10.5 to E12.5 (Gubbay et al., 1990; Koopman et al., 1990; Sinclair et al., 1990). According to the Sertoli cell organizer model, *Sry* should be expressed in Sertoli cells. However, since Sertoli cells can not be easily distinguished until approximately E12.5, it was not technically feasible to determine whether the differentiating Sertoli cell lineage expressed *Sry*. Evidence for this has only recently come to light with the creation of a line of transgenic mice that express GFP under the control of the *Sry* promoter (Albrecht and Eicher, 2001). The GFP expression of this transgene in XY genital ridges recapitulates the endogenous expression pattern that has been observed by whole-mount *in situ* hybridization (Bullejos and Koopman, 2001). However, the GFP expression from this transgene lasts somewhat longer than the endogenous expression. Observations in XY gonads at E12.5 revealed that the *Sry-GFP* transgene is only expressed in Sertoli cells. Unlike the endogenous *Sry* locus, the *Sry-GFP* transgene is not linked to the Y chromosome. Therefore this transgene is also expressed in XX genital ridges. In XX gonads the GFP expression is expressed until late in embryogenesis and is found in cells that have the morphological features of granulosa cells. This is suggestive evidence that

granulosa and Sertoli cells do share a common precursor. However, some caution in the interpretation of these results is warranted since under normal circumstances the *Sry*-promoter never encounters a feminized gonadal environment.

The germ cell and gonadal sex differentiaion

A common pattern has emerged from the study of early germ cell development in various vertebrate and invertebrate species (Fujimoto et al., 1975; Warrior, 1994). Primordial germ cells are allocated early in development and then migrate by various means from an extragonadal location to the gonads where they proliferate and differentiate. Primordial germ cells of the mouse are first detectable in extraembryonic tissue by specific alkaline phosphatase staining at E7-7.5 (Ginsburg et al., 1990). The primordial germ cells begin to actively migrate toward the genital ridges after E9.5, and most have reached the gonads by E11.5 (Tam and Snow, 1981). Throughout this period of migration the germ cells proliferate. After reaching the gonads, the germ cells continue to divide until approximately E13.5. Until this time male and female germ cells do not demonstrate any obvious histologically detectable sexual dimorphism (McLaren and Southee, 1997). At E13.5 male germ cells stop dividing and arrest as prospermatogonia, and female germ cells begin to enter meiotic prophase (Peters, 1970). Since male germ cells do not enter meiosis until approximately 10 days after birth, embryonic meiotic entry is considered to be the defining hallmark of female germ cell sex determination.

The presence of germ cells is not required for testis development (Handel and Eppig, 1979; Mintz and Russell, 1957). In mouse mutants that lack embryonic germ cells, the formation of the testicular cords may be somewhat delayed. However, testis

differentiation appears to be otherwise normal. Germ cell-less XY embryonic gonads form functional adult testes that can sustain spermatogenesis if wild-type donor spermatogonial stem cells are transplanted into the tubules (Brinster and Avarbock, 1994). It remains uncertain how the absence of germ cells affects early embryonic development of the ovary, but the presence of oocytes is clearly required later in embryogenesis for proper ovary differentiation (Adams and McLaren, 2002). As discussed above, embryonic ovaries appear to lack significant structural organization until late in embryogenesis. At around E18.5 the granulosa cells, also known as follicle cells, organize around and become tightly associated with oocytes. Each oocyte is surrounded by its own group of granulosa cells to form a follicle, and it is the follicles that are the basic structural feature of the ovary, much like the testicular cords are in the testis. In the absence of oocytes, follicles are not generated and the gonadal remnants form a "streak gonad" that consists primarily of fibrous connective tissue.

In mammals, germ cell sex is determined by tissue environment rather than by the sex chromosome constitution of the germ cell. In male chimeric mice containing XX and XY germ cells, XX germ cells in the testis develop as male prospermatogonia and stop undergoing cell division at ~E13.5 (Palmer and Burgoyne, 1991). Similarly, XX germ cells in XX sex-reversed mice initially develop as male (McLaren, 1981). However, certain Y chromosome genes are required to complete spermatogenesis (Mazeyrat et al., 2001). In addition, XX male germ cells die shortly after birth, possibly due to dosage problems associated with the presence of two X chromosomes in a male germ cell environment. However, it is clear that XX germ cells in a testicular somatic environment develop as male. In contrast, XY germ cells in female chimeras enter meiosis and

develop as oocytes (McLaren, 1984) as do XY germ cells in mouse models of XY sex-reversal (Taketo-Hosotani et al., 1989). Further evidence of somatic influences on germ cell sex differentiation has been obtained through the study of ectopic germ cells. These ectopic germ cells originate from a small percentage of primordial germ cells that do not properly complete their migration to the gonad. Ectopic XY germ cells which develop in the adrenal glands of male mice enter meiosis and develop into oocytes during the same period that germ cells in fetal ovaries do so (Zamboni and Upadhyay, 1983). This has provided evidence for what Byskov and Saxen have referred to as a "Meiosis Preventing Substance" (MPS) that is present in the fetal testis (Byskov and Saxen, 1976). These results do not rule out the presence of a meiosis promoting substance in the fetal ovary, but such a substance would also have to be present in the fetal adrenal gland as well.

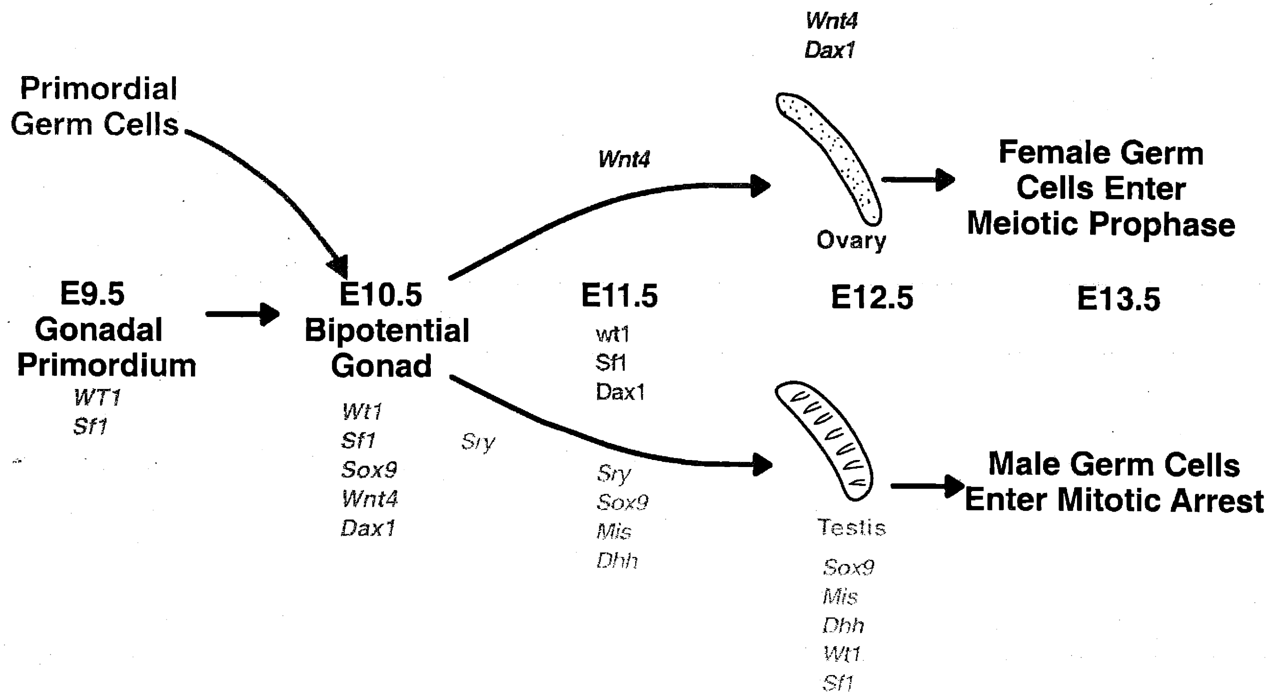
More recently McLaren reported tissue culture experiments in which primordial germ cells isolated from E11.5, E12.5, and E13.5 testes and ovaries were cultured with fetal lung tissue (McLaren and Southee, 1997). XX germ cells from all three periods entered meiosis. XY germ cells from E11.5 also entered meiosis, but XY germ cells isolated from E12.5 and E13.5 fetal testes developed as prospermatogonia. Similar results have been observed if testicular germ cells from these time periods are isolated and placed into reconstituted embryonic ovaries or grown on a monolayer of feeder cells (Adams and McLaren, 2002; Nakatsuji and Chuma, 2001). In a related series of experiments McLaren examined the ability of embryonic testes to masculinize XX ovarian germ cells (Adams and McLaren, 2002). E11.5 and E12.5 ovarian germ cells will develop as male prospermatogonia if placed into reconstituted embryonic testes. However, by E13.5 the majority of ovarian germ cells are committed to meiotic entry and

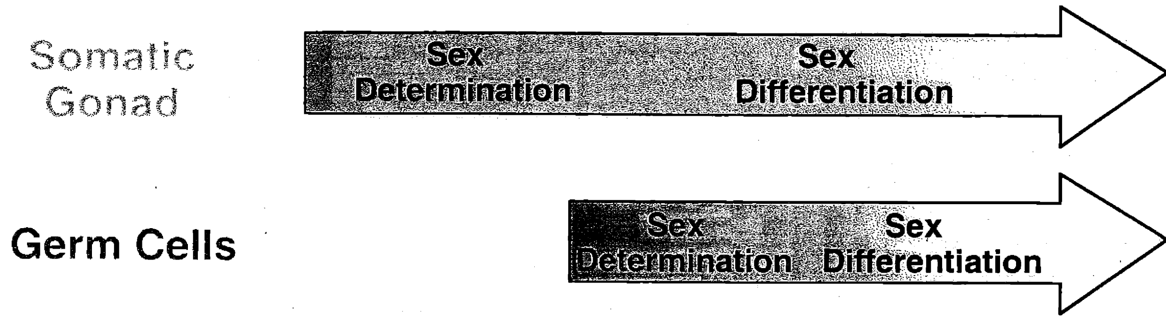
are not masculinized by a testicular environment. Therefore, germ cells in the embryonic testis are masculinized by E12.5 and germ cells in the embryonic ovary are committed to developing as female by E13.5. Furthermore, the observation that embryonic germ cells will enter meiosis in tissue culture suggests that embryonic germ cells may enter meiosis cell autonomously unless they develop in an embryonic testis.

Transcription factors of the somatic gonad

In most mammals the initiation of testis development is dependent upon the presence of the Y-linked gene *Sry* (Gubbay et al., 1990; Sinclair et al., 1990). The expression of *Sry* in the genital ridges of XX mouse embryos is sufficient to fully masculinize the gonad (Koopman et al., 1991). The protein encoded by the *Sry* gene contains an HMG box DNA binding domain and has the capacity to bind to and bend DNA (Werner et al., 1995). This property of SRY implies that it functions as transcriptional regulator. Similar DNA binding domains are found in a large group of SRY-related proteins known as the SOX (SRY-box containing proteins) family. Of the *Sox* genes, *Sox3* is the most closely related to *Sry*. *Sox3* itself is not thought to function in mammalian sex differentiation (Pask et al., 2000). However, since *Sox3* is X-linked, it is believed that *Sry* and *Sox3* originated from a common progenitor (Stevanovic et al., 1993). In this scenario the X and Y chromosomes were originally a normal pair of autosomes. It is thought that a few hundred million years ago a mutation occurred in *Sox3* that resulted in a testis determining activity. It is further believed that creation of this testis determining allele of *Sox3* was the event that initiated the formation of the proto Y chromosome.

Figure 2. Genes implicated in sex differentiation of the embryonic mouse gonad and their developmental expression. Many of these genes are initially expressed equally in XX and XY genital ridges (labeled in brown), before being expressed at higher levels in XX (red) or XY (blue) gonads.


Primordial
Germ Cells
E9.5
Gonadal
Primordium
WT1
Sf1
E10.5
Bipotential
Gonad
Wt1
Sf1
Sox9
Wnt4
Dax1
Sry
Wnt4
E11.5
wt1
Sf1
Dax1
Sry
Sox9
Mis
Dhh
Wnt4
Dax1
Ovary
E12.5
Testis
Sox9
Mis
Dhh
Wt1
Sf1
Female Germ
Cells Enter
Meiotic Prophase
E13.5
Male Germ Cells
Enter Mitotic Arrest


Somatic
Gonad
Sex
Determination
Sex
Differentiation
Germ Cells
Sex
Determination
Sex
Differentiation

The DNA binding properties of SRY imply that it functions as a transcriptional regulator, yet despite more than a decade of research no direct *in vivo* gene targets of SRY have been demonstrated. However, some potential targets of SRY have been identified. Undoubtedly the strongest candidate for direct regulation by SRY is another member of the *Sox* gene family, *Sox9*. *SOX9* was originally identified as the gene responsible for the human syndrome campomelic dysplasia (Foster et al., 1994; Wagner et al., 1994). Humans who are heterozygous for mutations in *SOX9* exhibit severe skeletal deformities and the majority of XY individuals develop as female. Duplication of the region containing *SOX9* has also been reported in an XX human male (Huang et al., 1999).

The form of XY sex-reversal observed in individuals with mutations in *SOX9* or *SRY* differs from many other XY sex-reversal syndromes in that the gonads are actually feminized. This should be distinguished from syndromes that disrupt gonad formation entirely. During testis development XY embryos normally produce male hormones including testosterone and Mullerian inhibiting substance (MIS). The production of testosterone in male embryos is required to masculinize the genitalia and to prevent the degeneration of the Wolffian ducts, which ultimately form the epididymis and the vas deferens. The male hormone MIS is required to cause the Mullerian ducts to regress. In the absence of MIS, the Mullerian ducts develop into the oviducts, the uterus and the upper portion of the vagina. Therefore, XY embryos that lack gonads will be phenotypically female even though this sex-reversal results from the loss of gonads rather than being a direct result of gonadal sex-reversal.

Unlike *Sry*, which is found only in mammals, *Sox9* is a well conserved gene that appears to exist in all vertebrates. In addition, *Sox9* is upregulated during embryonic testis development in a wide variety of species including chicken, gecko, alligator, and turtle (Choudhary et al., 2000; Morais et al., 1996; Moreno-Mendoza et al., 1999; Valleley et al., 2001; Western et al., 1999). In mice *Sox9* is expressed in the XY and XX bipotential gonad at E10 and is subsequently down regulated in XX and upregulated in XY genital ridges by E11.0 (Morais et al., 1996). At E12.5 *Sox9* expression is observed in Sertoli cells. Moreover, over expression of *Sox9* in XX mouse embryos results in complete masculinization of the gonad (Morais et al., 1996). Thus, in mammals the upregulation of *Sox9* by SRY could be sufficient to direct male development. SRY exhibits little conservation between different mammals with the exception of the HMG box, and all known XY sex reversal cases that are caused by mutations in SRY disrupt the HMG box. In order to investigate the specificity of the HMG box, Bergstrom et al. replaced the HMG box of *Sry* with that of *Sox3* or *Sox9* and generated transgenic mice (Bergstrom et al., 2000). Both the *Sox3/Sry* and the *Sox9/Sry* transgenes were able to effectively promote testis differentiation in XX mice. Unfortunately, the ability of these chimeric proteins and of SOX9 to mimic the effects of SRY is difficult to interpret with the currently limited knowledge of what DNA sites and what protein partners these molecules are interacting with *in vivo*.

In addition to SOX9, three other transcription factors have been proposed to participate in gonadal sex differentiation. These factors include WT1, SF1, and DAX1. The literature regarding these genes is extensive and sometimes conflicting. WT1 is a transcription factor that contains zinc finger DNA binding domains (Haber et al., 1990).

Like *SOX9* and *SRY*, *WT1* was first identified as the causal agent in human disease syndromes. Deletion or disruption of various *WT1* spliced transcripts in humans can cause a range of syndromes including predisposition for kidney tumors during childhood, mild to severe urogenital malformations and the occurrence of ambiguous or female genitalia in XY individuals (Denys-Drash and Frasier syndromes) (Klamt et al., 1998; Koziell et al., 2000; Koziell et al., 1999; Scharnhorst et al., 2001). Mice that are homozygous null for *Wt1* have arrested kidney development and lack gonads (Kreidberg et al., 1993). Since the deletion of *Wt1* results in the loss of embryonic gonads in male and female mice before sex differentiation occurs, the functional requirement for *Wt1* during testis differentiation could not be assessed in the original mouse knockout. However, more recent experiments that ablate particular splice variants have revealed different roles for two of the WT1 isoforms in gonad development (Hammes et al., 2001). These two variants result from alternative splicing of WT1 exon 9 that causes either the presence or absence of three amino acids (lysine, threonine, and serine; KTS) between the third and fourth zinc fingers of WT1. Ablation of the –KTS splice site results in the inability to produce the –KTS WT1 isoform. Similarly, ablation of the +KTS splice site prevents the production of +KTS WT1. Neither of these targeted mutations effect the overall level of transcription from the *Wt1* promoter. XY mice unable to produce +KTS WT1 exhibit reduced levels of *Sry* expression and XY gonadal sex reversal, while XX null mice contain normal appearing ovaries. In contrast, both XY and XX mice that are unable to produce –KTS WT1 have severe defects in gonadal development and the gonads of both sexes fail to differentiate. This suggests a requirement for the +KTS form

in testis development, while the –KTS form may have a more general role in male and female gonad development.

Experimental evidence has suggested that +KTS WT1 acts as a transcriptional repressor (Menke et al., 1998) while –KTS WT1 is a transcriptional activator (Lee et al., 1999). In fact, –KTS WT1 is capable of activating the expression of *Sry in vitro* while +KTS WT1 cannot (Hossain and Saunders, 2001). Based simply on this *in vitro* data one might conclude that +KTS WT1 is not required for the expression of *Sry*. This is contrary to the ablation study described above, in which loss of +KTS WT1 *in vivo* resulted in significantly reduced expression of *Sry*. This highlights the shortcomings of the *in vitro* cotransfection experiments upon which so many conclusions about SRY and other gonadal transcription factors have been based. Conclusions based on the over expression of transcription factors in an unnatural cellular context may not yield reliable insights or may be over interpreted unless coupled with *in vivo* experiments.

Yet another transcription factor that is believed to have role in gonadal sex differentiation is the orphan nuclear receptor steroidogenic factor 1 (SF1) (Nachtigal et al., 1998). SF1 was originally identified as a transcriptional activator of enzymes involved in steroid biosynthesis pathways (Ikeda et al., 1993). Targeted disruption of *Sf1* in mice results in gonadal degeneration in XX and XY embryos prior to sex differentiation and in the failure of the adrenal glands to develop (Luo et al., 1994). Thus, like the original *Wt1* knockout mouse the early gonadal requirement for *Sf1* prevents an analysis of possible later functions in testis or ovary development. *Sf1* is initially expressed in XX and XY gonads before being down-regulated in female gonads at E12.5, shortly after morphological differences between XX and XY gonads become apparent

(Ikeda et al., 1994). Early during testis differentiation SF1 is expressed by Sertoli and Leydig cells, though later its expression is lost in Sertoli cells. It will likely require the generation of a conditional *Sf1* allele to reveal later functional requirements for this gene in sex differentiation.

A second member of the nuclear receptor family, DAX1, is thought by many to be an "anti-testis" factor. *DAX1* is located in a region on the short arm of the human X chromosome that is associated with XY sex-reversal when duplicated (Zanaria et al., 1995). According to the proposed model, a double dosage of DAX1 in XY embryos inhibits testis development. This model has been partially substantiated through the generation of transgenic mice that over express *Dax1* (Swain et al., 1998). Normal XY mice do not exhibit sex reversal when *Dax1* is over-expressed. However, if the *Dax1* transgene is placed on a strain background that contains a weakly expressed *Sry* allele or transgene, testis development is inhibited. It is possible that the weak sex-reversing effect of the *Dax1* transgene is due to subtle differences between sex differentiation in mice in humans. Alternatively, DAX1 may not be the sole cause of the sex-reversal effect observed in humans. The duplicated region also contains members of the MAGE (Melanoma Antigen) family of proteins (Dabovic et al., 1995; Zanaria et al., 1995). The MAGE family is a large group of related proteins that are located on the X chromosome and that are expressed in various tumors as well as germ cells. No functional studies of the MAGE genes have been reported, likely due to the potentially redundant nature of this large family. More significant than the mild anti-testis effect of *Dax1* in mice is the phenotype of the *Dax1* knockout. *Dax1* null females are fully viable and fertile, while

Dax1 null males are sterile (Yu et al., 1998). Thus, this "anti-testis" gene is required for male fertility and its function is not required for ovary development in mice.

Secreted factors and signaling events in the differentiating testis

During the process of gonadal development, the embryonic testis and ovary must coordinate the differentiation of various somatic cell types and the germ cells. Several secreted testis factors have been identified. Some of these are required for testis differentiation and others are not. Perhaps somewhat surprisingly, the two "classic" male hormones, Mullerian inhibiting substance (MIS) and testosterone, are not required for testis differentiation. Testosterone is secreted by the Leydig cells of the testis and prevents the degeneration of the Wolffian ducts and promotes their differentiation into the epididymis and the vas deferens. Testosterone also induces male differentiation of the genitalia. In mice that carry the X-linked *Tfm* mutation, the androgen receptor is defective and the mice are incapable of responding to testosterone (He et al., 1991). Therefore the Wolffian ducts of XY *Tfm* mice degenerate and the external genitalia are feminized. Despite these defects, the gonads differentiate as testes and produce Mis. Meiotic germ cells are observed in the testes of XY *Tfm* mice, though they arrest in meiotic prophase.

Mullerian inhibiting substance (MIS) is a member of the TGFβ superfamily of secreted signaling molecules. Shortly after *Sry* and *Sox9* (Morais et al., 1996) are upregulated, the Sertoli cells begin to produce MIS . MIS causes the Mullerian ducts to regress, and thus prevents the formation of the uterus and oviducts in male embryos. Disruption of MIS in mice results in the retention of these female ducts in XY embryos (Behringer et al., 1994). Despite the absence of MIS and the presence of male and female reproductive ducts, XY MIS null mice develop normal testes with active

spermatogenesis. However, most of these male mice are infertile due to physical blockage of sperm passage caused by the presence of the female reproductive tract.

The Sertoli cells also express *Desert hedgehog* (*Dhh*) during embryonic testis development and into adulthood (Bitgood et al., 1996). *Dhh* is first detectable in XY genital ridges at E11.5, approximately the same that *Mis* is upregulated. DHH and the other members of the hedgehog family are secreted signaling molecules that are involved in a variety of different developmental processes during embryonic development. Targeted disruption of the *Dhh* gene results in male infertility, but does not effect the fertility of females (Bitgood et al., 1996). The severity of the *Dhh* null phenotype in males varies depending on the genetic background. In the least affected males, the seminiferous tubules are somewhat disorganized and largely devoid of germ cells. In these males a few tubules contain primary spermatocytes and some males have postmeiotic germ cells. The most severely affected XY individuals develop as pseudohemaphrodites with partially feminized external genitalia and extremely small undescended testes that lack germ cells (Clark et al., 2000). Observations of embryonic and juvenile *Dhh* null testes indicate that the loss of germ cells occurs postnatally. In addition, the germ cells are masculinized, as they do not enter meiosis prematurely. The hedgehogs modulate gene activity by inactivating the membrane bound Patched (PTC) receptor. Since *Ptc* is normally expressed in Leydig and peritubular myoid cells, the defects in cord organization, germ cell loss and testicular descent may stem from the inability of the Sertoli cells to communicate properly with these cell types (Bitgood et al., 1996; Clark et al., 2000). The *insulin-3* (*Insl3*) gene, which encodes another secreted factor of the testis, is normally upregulated in embryonic Leydig cells beginning at E13.5

(Nef and Parada, 1999; Zimmermann et al., 1999). Since the secretion of the INSL3 protein is required for trans-abdominal descent of the testes during embryonic development it is possible that the absence of DHH signaling could effect Leydig cell secretion of INSL3. Regardless of the precise cascade of events, it is clear that embryonic testis differentiation is perturbed in the absence of DHH.

A much more severe disruption of testis differentiation has been observed in mice deleted for *Fibroblast growth factor 9* (*Fgf9*) (Colvin et al., 2001). *Fgf9* is expressed in a wide variety of tissues, but during early gonadal development it is expressed in XY but not XX genital ridges. *Fgf9* null mice die shortly after birth due to lung hypoplasia (Colvin et al., 2001), but during embryonic development XX embryos undergo normal ovary development. In contrast, almost all XY null embryos exhibit XY sex-reversal and develop ovaries (Colvin et al., 2001). In these sex-reversed XY ovaries, the expression of Sertoli cell and Leydig cell genes is confined to small regions or is completely absent. *In vitro* organ culture experiments have demonstrated that addition of FGF9 to culture medium can induce migration of mesonephric cells into XX gonads. It has therefore been proposed that FGF9 may be required for the Sertoli cells to recruit additional cells from the mesonephros. As discussed earlier, migration of cells from the mesonephros is required for testicular cord formation. Therefore, the inability to recruit mesonephric cells could result in the failure of testis differentiation that is observed in *Fgf9* -/- XY mice.

Secreted factors and signaling events in the differentiating ovary

The great majority of embryonic sexually dimorphic molecules that have been described in the literature are testis genes rather than ovary genes. This may be due in part to the large changes in gene expression that are required to support the sudden and

extensive structural reorganization that occurs during early testis differentiation. However, it seems quite likely that this lack of ovary genes is also due to the focus on testis differentiation rather than ovary differentiation. The strong focus on testis differentiation has probably occurred for the simple reason that the structural changes are so dramatic and the differentiation of distinct testicular cell types occurs much earlier than the morphologic differentiation of ovarian cell types. In addition, embryonic ovarian differentiation and female development in general has often been thought of as a default pathway, rather than as the active developmental process that it is.

Despite our more limited knowledge of ovarian factors, a few insights into ovary development have been gained recently through the phenotypic consequences of different mouse knockouts. Targeted disruption of *Wnt4*, a member of the WNT family of secreted signaling molecules, results in kidney failure, and death follows shortly after birth (Vainio et al., 1999). In addition to kidney defects, *Wnt4* -/- XX embryos have partially masculinized gonads and lack Mullerian ducts. By birth, *Wnt4* null ovaries contain occasional cord-like structures and have few germ cells. XY null gonads appear to undergo normal testis development. An expression analysis revealed the ectopic production of male steroid producing enzymes in the embryonic ovaries of *Wnt4* null XX gonads. These enzymes are normally expressed in the male Leydig cells. A further analysis revealed that Sertoli cell genes are not expressed in XX null ovaries during early gonad differentiation, though they are expressed by birth. Therefore, *Wnt4* expression in embryonic ovaries appears to be required to repress Leydig cell differentiation, but is not required to repress Sertoli cell development during the initial stages of gonad development. WNT4 may also be required for oocyte development, since the germ cells

of XX gonads die during late embryogenesis. However, it is unclear whether this oocyte loss is due to a direct requirement for WNT4 or results less directly from the presence of masculinized somatic cells in the ovary. WNT4 may play a role in human sex-reversal as well. Duplications of human chromosome 1p31-35 are associated with XY sex-reversal and *Wnt4* lies within this region (Jordan et al., 2001). It has been proposed that over expression of Wnt4 may contribute to the phenotype observed in these individuals.

The testis cord-like structures that occur in *Wnt4* -/- XX gonads do not form until after the oocytes are lost (Vainio et al., 1999). This is consistent with other evidence that shows the presence of oocytes may promote female differentiation of somatic cells and inhibit male differentiation (Behringer et al., 1990; Taketo et al., 1993). The most striking example of this has been observed in double knockout mice that lack both α and β estrogen receptors (αβER null mice) (Couse et al., 1999). Prepubertal female αβER null mice have large adult-like follicles. The elevated levels of gonadotropins that result from an inability to respond to estrogen are the likely cause of this precocious growth. The ovaries of adult αβER null mice contain some growing follicles, but also display structures that strongly resemble seminiferous tubules. These structures make up a large fraction of αβER null adult ovaries and degenerating oocytes can be observed in some of these tubule-like structures. The lack of these structures in juvenile mice, their age dependent increase, and the spherical shape of these structures suggest that they originate from follicles in which the oocyte has died. Cells that contain the morphological features of Sertoli cells are present in the tubule-like structures and αβER null ovaries contain elevated levels of Sertoli cell genes including *Sox9* and *Mis*. This apparent example of sex-reversal is remarkable in that it occurs long after differentiation of the embryonic

gonad takes place and occurs in ovaries that initially appear to be fully differentiated as female. It is unlikely that the sex-reversal observed in αβER null ovaries is caused simply by oocyte death since there are several mouse knockouts that result in oocyte death at both early and late time points that do not result in the formation of tubules. αER is normally expressed in thecal cells while βER is produced by the granulosa cells. Therefore, loss of these estrogen receptors may predispose the somatic cells of the ovary to redifferentiate as male in the absence of oocytes. Alternatively, it may be the differentiation of ovarian somatic cells as male that results in oocyte death. While there is no evidence that estrogen synthesis is required for embryonic development of the mammalian ovary, estrogen synthesis is important for ovarian development in nonmammalian vertebrates (Western and Sinclair, 2001). The mechanisms involved in postnatal sex reversal of αβER null mouse ovaries may be related to the estrogen requirement in these species.

Problems and progress in understanding sex differentiation

From the body of knowledge that has been gathered regarding gonadal sex differentiation, it has become clear that this is multi-step process. The expression of *Sry* during a small window of time in a specific subset of somatic cells is sufficient to initiate Sertoli cell development. This is generally considered the sex-determining event, since under normal circumstances differentiation of the Sertoli cells is sufficient to direct differentiation of the gonad towards a testicular fate and away from an ovarian fate. Yet the reality is that multiple events must occur for proper differentiation to occur.

The mechanisms underlying mammalian gonadal sex differentiation have remained somewhat elusive. One reason for this has been the lack of clear answers from

mouse knockouts of sex differentiation candidate genes. The *Sf1* knockout (Luo et al., 1994) and the original *Wt1* knockout (Kreidberg et al., 1993) revealed the functional requirement for these genes in gonad development prior to sex differentiation, but were not informative with regards to later functions. Disruption of *Sox9* in mice is lethal in the heterozygous state and XY heterozygous animals have normal appearing testes (Bi et al., 1999; Bi et al., 2001). Moreover, *Sox9* null mice that are generated by tetraploid blastocyst injections die at E11.5 before sex differentiation occurs. Though over expression of *Sox9* can masculinize the gonads of XX mice (Vidal et al., 2001), a conditional knockout will be required to determine the requirement for Sox9 during testis development. In contrast to the knockouts of *Sf1*, *Wt1* and *Sox9*, the knockout of *Dax1*, the "anti-testis" gene, seems to have given a clear but unexpected answer. *Dax1* is not required for ovary differentiation, at least in mice, but is required for male fertility (Yu et al., 1998). Somewhat surprisingly, the most informative knockouts have come from genes that were not previously implicated in gonad development. Loss of *Wnt4* perturbs ovary development by allowing the formation of Leydig cells (Vainio et al., 1999). The *Fgf9* knockout disrupts testis development, possibly by preventing the Sertoli cells from recruiting cells from the mesonephros (Colvin et al., 2001).

The study of gonadal sex differentiation has also been slowed by the dependence on human sex-reversal syndromes as a source of candidate genes. The analysis of human sex-reversal patients led to the identification of *SRY* (Sinclair et al., 1990), *SOX9* (Foster et al., 1994; Wagner et al., 1994), *WT1* (Haber et al., 1990), and *DAX1* (Zanaria et al., 1995). However, the heterogeneous nature of the molecular causes of XY and XX sex-reversal makes the identification of additional candidate genes difficult. Recent efforts to

identify the gene expression differences that develop between embryonic testes and ovaries in the mouse have begun to yield new candidate genes (Bowles et al., 2000; Grimmond et al., 2000; Perera et al., 2001; This Thesis). While functional analysis of these new genes is still lacking, this line of work promises to provide a more complete picture of gonadal sex differentiation.

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Chapter 2

Sex Specific Gene Expression Differences in the Developing Mouse Gonad

Douglas B. Menke and David C. Page

Abstract

Many fundamental aspects of mammalian sex differentiation remain to be elucidated. Additional components of the testicular and ovarian developmental pathways must be identified to understand this process. I have investigated the expression differences that underlie the process of gonadal sex differentiation in the mouse through the use of a modified PCR based subtraction. Subtraction of embryonic day 12.5 (E12.5) XY gonadal cDNA with E12.5 XX gonadal cDNA yielded fourteen genes that are present in embryonic XY gonads at significantly higher levels than embryonic XX gonads. These genes include *α-dystrobrevin*, *hedgehog-interacting protein*, *osteopontin*, *p21-activated kinase 3*, *cerebellin 1*, *Cyp26b1*, and an additional eight novel genes. These genes display a variety of different expression patterns within the embryonic testis and encode a range of different protein classes. In addition, I performed a reciprocal ovarian subtraction in which I successfully identified two genes, *follistatin* and *Adamts19*, that are expressed at higher levels in ovaries than testes. Follistatin is a well-known antagonist of TGFβ family members while *Adamts19* encodes a new member of the ADAMTS family of secreted metalloproteases.

Introduction

In mammals, the sexual fate of the organism is ultimately dependent upon the expression of the testis-determining gene, *Sry*, in the embryonic gonad. It is believed that the expression of *Sry* induces a subset of gonadal cells to differentiate as Sertoli cells (Albrecht and Eicher, 2001; Koopman et al., 1990). Following this event, the Sertoli cells direct the recruitment and differentiation of additional testicular cell types (Capel et al., 1999). In the absence of SRY, Sertoli cell differentiation is not initiated and the bipotential gonad develops into an ovary.

Our ability to understand the intricacies of gonadal sex differentiation is in large part linked to our knowledge of the gene expression differences that exist between the differentiating testis and ovary. *Sry* is expressed in the XY genital ridge of the mouse embryo from approximately E10.5 to E12.5 (Koopman et al., 1990). The peak of *Sry* expression occurs at E11.5. This is the earliest stage at which additional gene expression differences have been detected between differentiating XX and XY genital ridges, though it is not until E12.5 that XX and XY gonads can be macroscopically distinguished. A relatively small number of gene expression differences have been identified during these early stages of gonadal sex differentiation. Moreover, of the differentially expressed transcripts that have been described, most are testis genes that are expressed in Sertoli cells. We are largely ignorant of changes in gene expression that occur during ovary development and that occur during the development of other testicular cell lineages including the germ cells, the steroid producing Leydig cells, and the peritubular myoid cells.

The identification of mammalian genes required for gonadal sex differentiation has occurred primarily via two distinct routes. The first of these is through the identification of genes involved in human sex reversal syndromes, as with *SRY* (Sinclair et al., 1990), *SOX9* (Foster et al., 1994; Wagner et al., 1994), *WT1* (Pelletier et al., 1991), and *DAX1* (Bardoni et al., 1994). The second means of identification has been through

the observance of gonadal defects resulting from the functional disruption of mouse genes that were not previously suspected to be involved in sex differentiation, as with *Wnt4* (Vainio et al., 1999) and *Fgf9* (Colvin et al., 2001). To identify additional transcripts that are expressed in a sexually dimorphic pattern and that may be involved in the differentiation of testicular and ovarian cell lineages, I have undertaken an expression based approach.

Results

Isolation of testis genes

In order to discover gene expression differences between differentiating embryonic testes and ovaries, I performed two PCR based cDNA subtractions. I began by subtracting E12.5 XY gonadal cDNA with E12.5 XX gonadal cDNA to generate a cDNA pool enriched for testis transcripts. E12.5 is the earliest time period at which XX and XY gonads can be visually distinguished, and one would expect numerous differentially expressed genes to be present at this developmental stage. After four rounds of subtraction, I cloned the resulting cDNA fragments and randomly selected 1152 cDNA clones for sequencing. Of these sequenced clones, quality sequence reads were obtained for 1078. Strikingly, 30% of the cDNA clones originated from four different Y chromosome genes (*Smcy*, *Uty*, *Dby*, and *Eif2s3Y*; Table 1). These Y chromosome genes are ubiquitously expressed in male tissues and, of course, are completely absent in females (Agulnik et al., 1994; Ehrmann et al., 1998; Greenfield et al., 1996; Mazeyrat et al., 1998). This demonstrates the dramatic enrichment of differentially expressed genes achieved in my subtraction. An additional 24% of the cDNA clones represent many of the genes previously reported to be upregulated during testis differentiation. These genes include *Mullerian inhibiting substance* (*Mis*) (Munsterberg and Lovell-Badge, 1991), *desert hedgehog* (*Dhh*) (Bitgood et al., 1996), *17-α-hydroxylase* (*Cyp17*) (Greco and Payne, 1994), and *testatin* (Tohonen et al., 1998), as well as several other genes that were identified independently in this work and that have been recently reported elsewhere: *protease nexin-1* (*Serpine2*) (Grimmond et al., 2000), *renin* (Perera et al., 2001), *collagen type IX α 3* (*Col9a3*) (Perera et al., 2001), *prostaglandin D2 synthase* (*Ptgds*) (Adams and McLaren, 2002), and *cadherin 11* (*Cdh11*) (Wertz and Herrmann, 2000).

The representation of different genes in my subtracted cDNA library varied dramatically. For instance, *Dhh* and *renin* were each represented by ~100 clones (9%) while *Mis*, *protease nexin-1*, and *Ptgds* were represented by 2 clones each (<0.2%).

These differences are undoubtedly due to a combination of the initial abundance of these transcripts in embryonic gonads and PCR amplification biases. In addition, I found that the majority of my cDNA fragments are derived from 3' UTR sequences rather than coding sequence. Certain genes that are known to be differentially expressed at E12.5 were not present amongst the sequenced cDNAs. Notably, *Sox9*, *vanin 1*, and *tescalcin* were absent (Bowles et al., 2000; Grimmond et al., 2000; Morais et al., 1996; Perera et al., 2001). However, the fact that particular transcripts were only recovered twice out of 1078 sequenced clones, suggests that additional sequencing efforts may reveal more of these previously identified genes.

After removing Y chromosome genes and other previously reported differentially expressed genes, I was left with 631 sequences. 73 of these sequences corresponded to housekeeping genes (e.g., rRNAs, mitochondrial genes, and ribosomal proteins) and were discarded. Of the remaining 558 sequences, I examined the expression of all novel sequences that were represented by two or more cDNA clones. I also examined a select number of known genes that were represented by multiple clones and/or that were deemed unlikely to be ubiquitously expressed based on BLAST searches against the NCBI EST database. I initiated my expression analysis by performing RT-PCR on testes and ovaries at E12.5, E13.5, and E14.5 (Fig. 1). However, because most of the sequences that I tested were differentially expressed (~80%) I performed subsequent expression analysis by *in situ* hybridization and shifted my efforts to obtaining full length coding sequence for novel differentially expressed genes.

I ultimately identified nineteen genes that are upregulated in embryonic XY gonad relative to embryonic XX gonad at E12.5 (Table 1). Five of these genes have recently been reported elsewhere (see references in Table 1). The differential expression of fourteen of these transcripts has not been previously reported. These fourteen genes include six known genes and eight novel genes (Table 1 and *Discussion*). Whole-mount

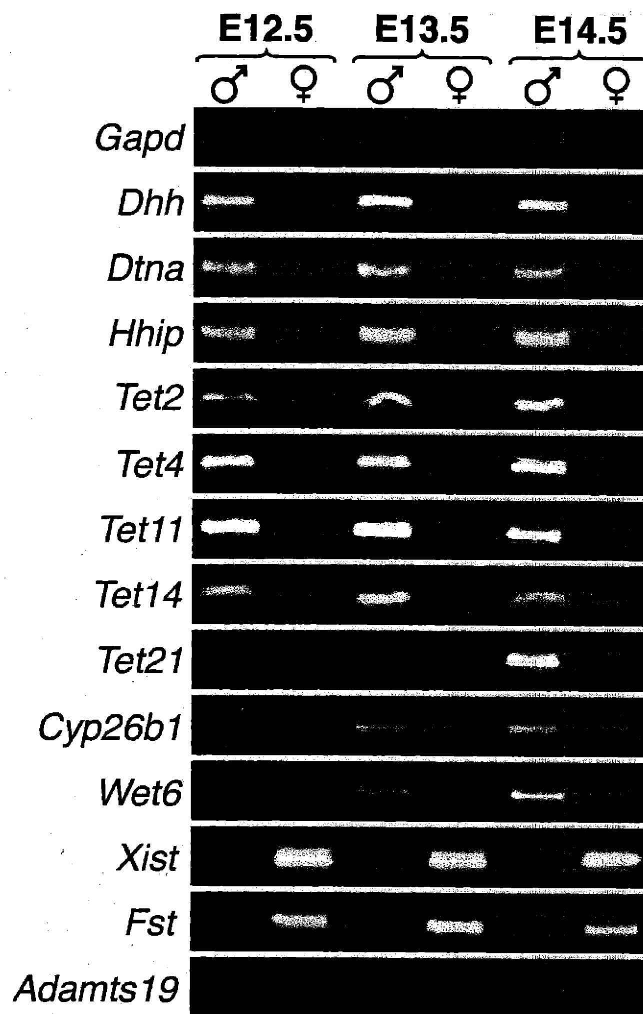
Table 1. Testis Subtraction

Gene Symbol	Gene Name	Number of Clones*	Expression	Comments/References
Dby	*DEAD box, Y*	137	Y	(Mazeyrat et al., 1998)
Eif2s3y	*Eukaryotic translation initiation factor 2, subunit 3, Y*	100	Y	(Mazeyrat et al., 1998)
Uty	*Ubiquitously transcribed tetratricopeptide repeat, Y*	53	Y	(Greenfield et al., 1996)
Smcy	*Selected mouse cDNA, Y*	32	Y	(Agulnik et al., 1994)
Dhh	*Desert hedgehog homolog*	95	C	(Bitgood et al., 1996)
Cyp17	*Cytochrome P450 17-α hydroxylase/C17-20 lyase*	23	I	(Greco and Payne, 1994)
Cst9	*Cystatin 9 (testatin)*	5	C	(Tohonen et al., 1998)
Mis	*Mullerian inhibiting substance*	2	C	(Munsterberg and Lovell-Badge, 1991)
Ren1	*Renin*	100	I	This study and (Perera et al., 2001)
Col9a3	*Procollagen, type IX, α 3*	33	C	This study and (Perera et al., 2001)
Serpine2	*Serine (or cysteine) proteinase inhibitor, clade E, member 2 (protease nexin 1)*	2	C	This study and (Grimmond et al., 2000)
Ptgds	*Prostaglandin D2 synthase*	2	C	This Study and (Adams and McLaren, 2002)
Cdh11	*Cadherin 11*	1	I,M	This study and (Wertz and Herrmann, 2000)
Dtna	*α-Dystrobrevin*	17	C	(Grady et al., 1999)
Hhip	*Hedgehog interacting protein*	11	I	(Chuang and McMahon, 1999)
Spp1	*Secreted phosphoprotein 1 (osteopontin)*	10	C,D	(Liaw et al., 1998)
Cyp26b1	*Cytochrome p450, subfamily 26b, polypeptide 1*	6	C,I	(White et al., 2000) Retinoic acid hydroxylase
Pak3	*p21-activated kinase 3*	3	C	(Allen et al., 1998)
Cbln1	*Cerebellin 1 precursor protein*	38	I	(Urade et al., 1991)
Cbln4	*Cerebellin 4 precursor protein* Novel	17	C	New cerebellin family member
Tet2	Novel	80	C	83a.a., β-Defensin Related
Tet4	Novel	47	C	167a.a., No homology
Tet11	Novel	18	I	G-coupled receptor, partial sequence, 79% identical to Gpr73
Tet14	Novel	7	C	59a.a., No homology
Tet21	Novel	4	I	206a.a., 39% identical to Sclerostin
Tet39	Novel	4	C	247a.a., 69% identical to Mmd
Wet6	Novel	9	C	390a.a., Contains ankyrin repeats

* A total of 1078 clones were sequenced.

Shaded region includes genes previously reported to be differentially expressed at higher levels in embryonic testes than embryonic ovaries. Y=Ubiquitous Y chromosome gene, C=Testicular cords, I=Interstitial cells, M=mesonephros, D=Mullerian duct

Figure 1. RT-PCR expression analysis of testis and ovary subtraction products on E12.5, E13.5 and E14.5 testis and ovary. *Gapd* served as a testis/ovary common control. *Dhh* and *Xist* served as testis and ovary controls respectively.


E12.5
E13.5
E14.5
♂
♀
♂
♀
♂
♀
Gapd
Dhh
Dtna
Hhip
Tet2
Tet4
Tet11
Tet14
Tet21
Cyp26b1
Wet6
Xist
Fst
Adamts19

in situ hybridization of E13.5 testes and ovaries revealed the expression of these genes in different cell types. Of the original nineteen genes, most are expressed within the testicular cords (Table 1 and Fig. 2). One of these, *osteopontin*, is also expressed in the Mullerian duct of male and female embryos, and appears to be more abundant in the Mullerian ducts of female embryos than male embryos at E13.5 (Fig. 2E). Another gene, *Cyp26b1*, is detectable primarily in the testicular cords but is also expressed outside the cords in a small number of interstitial cells (Fig. 2 Q). The remaining genes are expressed outside the testicular cords, though it is evident from their different expression patterns that these genes are expressed in different subsets of cells (compare Fig. 2M, N, O, P, Q, R, and S).

Isolation of ovary genes

Having succeeded with my testis subtraction, I performed a reciprocal experiment in which I subtracted E12.5 XX gonadal cDNA with E12.5 XY gonadal cDNA. After four rounds of subtraction, I randomly chose 192 clones for sequencing of which 188 produced quality data. Approximately 54% of these cDNA clones originated from Xist RNA (Table 2). Due to this extreme abundance of Xist, I sequenced fewer clones from the ovarian subtraction than for the testis subtraction. The non-coding Xist RNA is abundantly expressed from the inactive X chromosome of female cells and is not present at significant levels in males (Brown et al., 1991). Thus, the extreme enrichment of *Xist* in my ovarian subtraction is related to dosage compensation rather than to an ovary specific function. Included among the genes known to be upregulated in the E12.5 XX gonad are *Wnt4*, *Dax1*, *Gpbox*, *Psx1*, and *Gata2* (Siggers et al., 2002; Swain et al., 1998; Takasaki et al., 2000; Vainio et al., 1999). The overwhelming presence of *Xist* negatively impacted my ability to identify differentially expressed ovarian genes. However, the isolation of a single *Wnt4* cDNA clone indicated that other differentially expressed genes were represented in my subtracted library.

Figure 2. Whole-mount *in situ* hybridization of differentially expressed testis genes on E13.5 testes and ovaries. A) *Col9a3*, B) *Ptgds*, C) *protease nexin 1*, D) *Dtna*, E) *osteopontin*, arrows indicate expression in mesonephros, F) *Pak3*, G) *Cbln4*, H) *Tet2*, I) *Tet4*, J) *Tet14*, K) *Tet39*, L) *Wet6*, M) *Cadh11*, N) *Hhip*, O) *Cbln1*, P) *Tet11*, Q) *Cyp26b1*, R) *Tet21*, and S) *Ren1*. In all panels embryonic testes are located on the left and embryonic ovaries on the right.

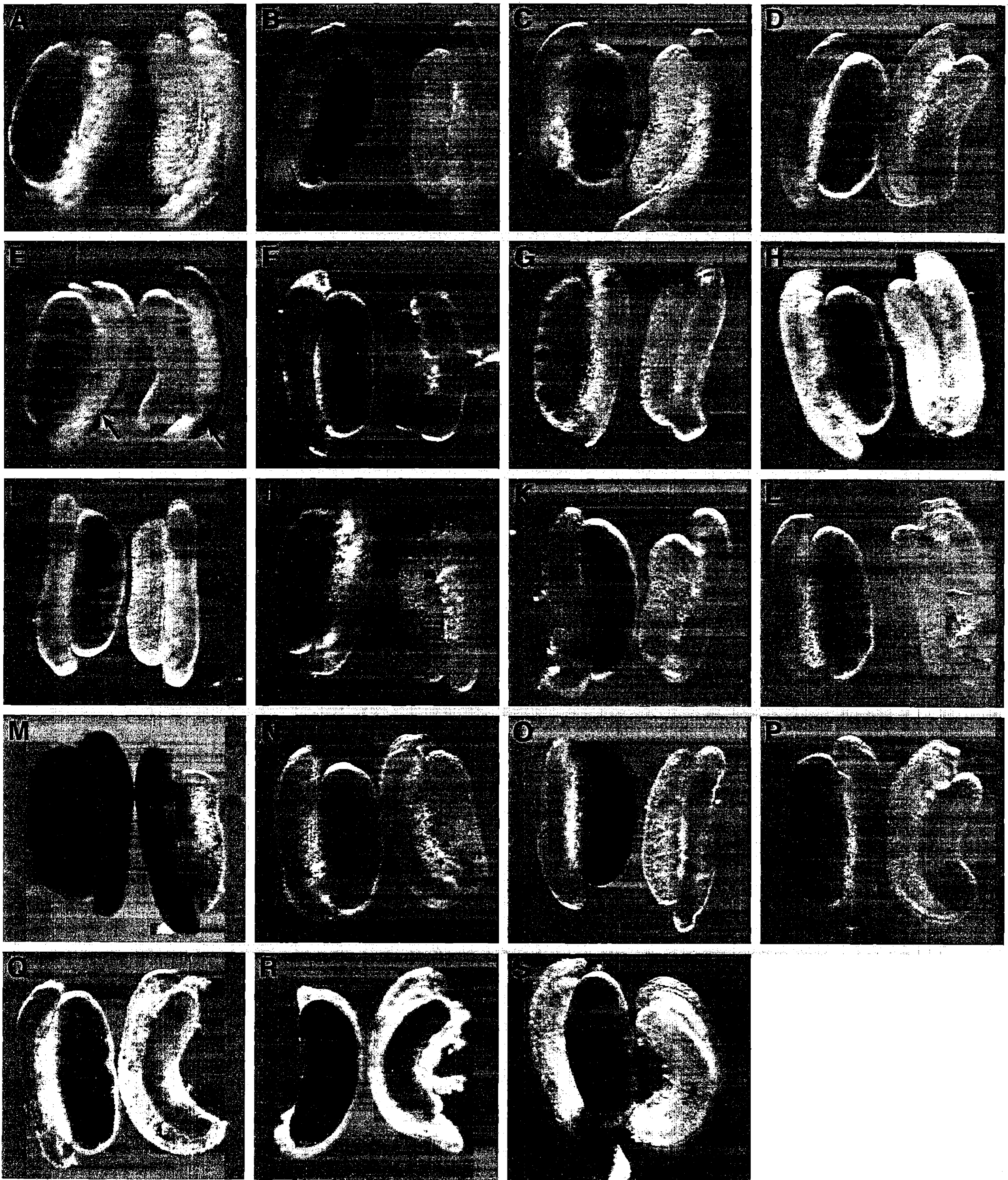


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Table 2. Ovary Subtraction

Gene Symbol	Gene Name	Number of Clones*	Comments/References
Xist	*Inactive X specific transcript*	101	Female dosage compensation (Brown et al., 1991)
Wnt4	*Wingless-related MMTV integration site 4*	1	Required for ovarian development (Vainio et al., 1999)
Fst	*Follistatin*	13	TGFβ binding protein (Matzuk et al., 1995)
Adamts19	*A disintegrin-like and metallo-protease (reprolysin type) with thrombospondin type 1 motif, 19*	1	New member of the ADAMTS family of metalloproteases

*A total of 188 clones were sequenced. Shaded region includes genes previously reported to be differentially expressed at higher levels in embryonic ovaries than embryonic testes.

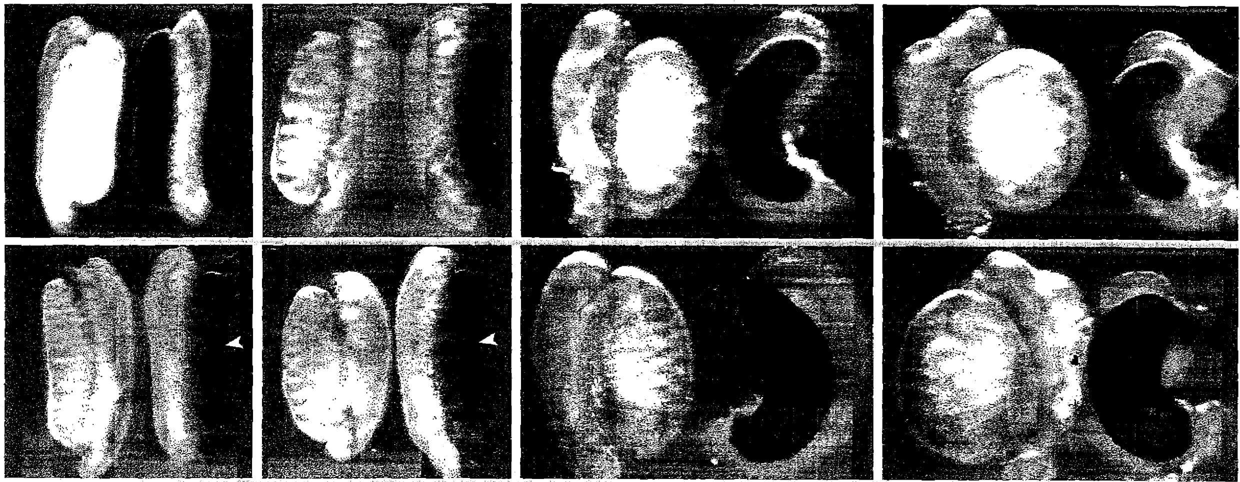
Other than *Xist*, the only non-housekeeping gene represented by multiple cDNA clones in my ovarian subtraction was *follistatin* (Table 2). Follistatin functions as an antagonist of TGFβ family members and is capable of binding to and inhibiting the function of activins and various BMPs (reviewed in (Patel, 1998)). Examination of *follistatin* expression by RT-PCR confirmed that *follistatin* is more abundant in embryonic ovaries than embryonic testes at E12.5, E.13.5, and E14.5 (Fig. 1). Encouraged by these results, I tested ten novel sequences from my subtraction for differential expression. One of these ten sequences was clearly present at higher levels in embryonic ovaries (Fig. 1 bottom, and data not shown). I performed 5' and 3' RACE to obtain the entire coding sequence of this transcript and found that this cDNA is derived from *Adamts19*, a new member of the ADAMTS (a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif) family of secreted metalloproteases (Tang, 2001).

Whole-mount *in situ* hybridization confirmed that both *follistatin* and *Adamts19* are more abundant in embryonic ovaries than embryonic testes from at least E12.5 to E15.5 (Fig. 3). At E12.5 and E13.5, *Adamts19* expression was strongest in anterior and ventral regions of the ovary (Fig. 3E and F). By E14.5 *Adamts19* appeared to be present in an even distribution across the ovary (Fig3 G and H). Over the same developmental time period *follistatin* displayed intense uniform staining in ovaries at all stages (Fig. 3A, B, C, and D). No *follistatin* expression was detected in testes. However, faint *Adamts19* expression was evident in embryonic testes at E14.5 and E15.5 (Compare Fig. 3C and G, and Fig. 3D and H).

Expression in other tissues and in germ cells

Though all the genes displayed in Tables 1 and 2 are differentially expressed between embryonic testes and ovaries, I also wanted to know whether the genes identified here are expressed in other tissues. Previous reports have demonstrated that

Figure 3. Whole-mount *in situ* hybridization of differentially expressed ovary genes in embryonic gonads from E12.5 to E15.5. *Follistatin* expression at A) E12.5, B) E13.5, C) E14.5, and D) E15.5. *Adamts19* expression at E) E12.5, F) E13.5, G) E14.5, and H) E15.5. In all panels embryonic testes are located on the left and embryonic ovaries on the right. Black arrows in panels E) and F) indicate the anterior tip of the ovaries, white arrowheads indicate the ventral surface of the ovaries.



dtna, *osteopontin*, *Pak-3*, *Cbln1*, *Hhip*, *Cyp26b1* are not specifically expressed in testes (Abu-Abed et al., 2002; Holzfeind et al., 1999; Manser et al., 1995; Oldberg et al., 1986; Satoh et al., 1997; Urade et al., 1991). To examine the testis specificity of the remaining genes, I performed RT-PCR on a panel of cDNAs generated from 12 different tissues. Eleven of these tissues were derived from eight day postpartum (8pp) mice and one from germ cell depleted adult testis. My results show that only a few of the genes isolated in my testis subtraction display testis specificity (Fig. 4). *Tet2*, *Tet39*, and *Wet6* all are expressed in ovary and other tissues in addition to testis by 8pp, while *Cbln4* is expressed in brain and testis and *Tet21* is found primarily in kidney. Moreover, I detected low levels of *Tet4* in most tissues (data not shown), and found many brain ESTs for this gene. *Tet14* is the only gene for which I have found no evidence for expression outside the testis. Consistent with the testis specificity of *Tet14*, additional RT-PCR performed on E9.5 and E10.5 embryos failed to reveal any *Tet14* expression.

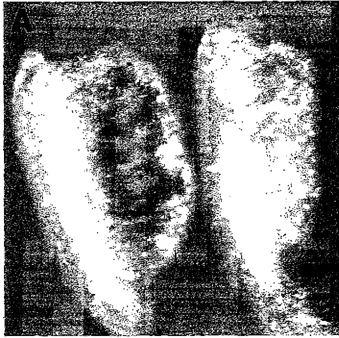
Among the tissues that I included in my RT-PCR analysis is testis from adult W^v/W^v mutant mice. W^v mice contain a point mutation in the tyrosine kinase receptor c-kit and are severely depleted of germ cells (Nocka et al., 1990). Therefore, the strong RT-PCR signal observed for most of my genes in germ cell depleted testes suggests that the expression of these genes is largely independent of germ cells. To confirm these results I tested a subset of the testis genes for expression in busulfan treated embryonic gonads by whole-mount *in situ* hybridization. Exposure of embryos to busulfan virtually eliminates the presence of germ cells (Merchant, 1975). *Cbln1* is expressed intensely in somatic cells outside of the cords and its expression is unlikely to be influenced by the absence of germ cells (Fig. 2 O). In agreement with my expectations, busulfan treatment does not cause a noticeable reduction in *Cbln-1* expression (Fig. 5D). *Cbln4*, *Tet2*, and *Tet4* are all expressed within the testicular cords of both untreated and busulfan treated E13.5 testes (Fig. 2G, H, and I; Fig. 5A, B, and C). Since there are both Sertoli cells and germ cells in

Figure 4. RT-PCR expression analysis of novel testis and ovary genes on 8 day post partum mouse tissues and germ cell depleted W^v/W^v adult testes. *Gapd* served as a ubiquitous control.

Brain
Kidney
Thymus
Skin
Heart
Skeletal Muscle
Lung
Wv/Wv Testis
Liver
Ovary
Spleen
Testis

Gapd
Cbln4
Tet2
Tet4
Tet13
Tet14
Tet21
Tet39
Wet6
Adamts19

Figure 5. Expression of testis and ovary genes in busulfan treated E13.5 testes and ovaries. Whole mount in situ hybridization of A) *Cbln4*, B) *Tet2*, C) *Tet4*, D) *Cbln1*, E) *follistatin*, and F) *Adamts19*. In all panels embryonic testes are located on the left and embryonic ovaries on the right.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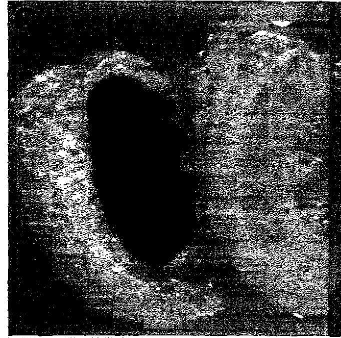
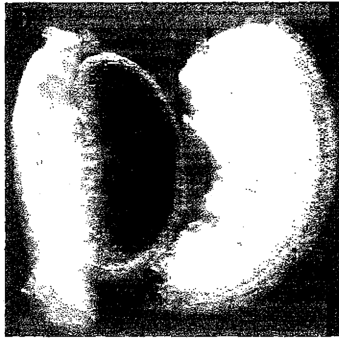
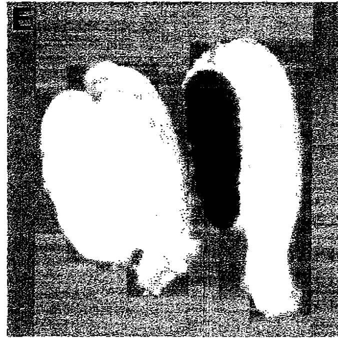
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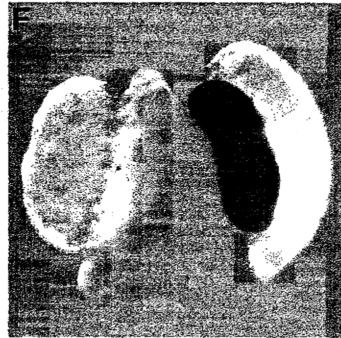
F

the cords, the presence of these transcripts in busulfan treated gonads indicates that the most likely source of expression is Sertoli cells.

Adamts19, the only novel differentially expressed gene isolated in my ovarian subtraction, is most abundantly expressed in ovary at 8pp (Fig. 4). Low levels of expression are also observed in kidney, heart, skeletal muscle, lung, and testis. *Follistatin*, the second ovarian gene I identified, is known to be expressed in a wide variety of tissues both embryonically and postnatally (Albano et al., 1994; Feijen et al., 1994). By whole-mount in situ hybridization *follistatin* produces a uniform staining in E13.5 ovaries, while *Adamts19* staining shows anterior-posterior and ventral-dorsal gradients (Fig. 3). Neither of these patterns resembles the punctate pattern that is generally observed with germ cell specific genes at these developmental stages (my personal observation). To demonstrate the germ cell independent expression of these genes, I examined their expression in busulfan treated gonads. I observed intense staining for both *Adamts19* and *follistatin* (Fig. 5E and F). Thus, the expression of these genes is not dependent on the presence of germ cells.

Discussion

Ovarian Factors

I have succeeded in identifying a large number of genes that demonstrate sexually dimorphic expression patterns during the differentiation of embryonic mouse gonads. Of particular interest is my identification of two molecular markers of ovarian differentiation, *Adamts19* and *follistatin*. Both *Adamts19* and *follistatin* are somatically expressed in embryonic ovaries (Fig 5E and F). *Follistatin* encodes a secreted protein that is capable of binding to and antagonizing the function of multiple members of the TGFβ family including the activins and certain BMPs (Patel, 1998). *Follistatin* is expressed at numerous sites both embryonically and postnatally including the granulosa cells of the ovary (Shintani et al., 1997; Sidis et al., 1998), and its expression in embryonic gonads has been previously reported (Feijen et al., 1994). However, it was not previously appreciated that *follistatin* expression is sexually dimorphic between embryonic ovaries and embryonic testes. Disruption of the *follistatin* gene in mice results in pleiotropic effects and death ensues shortly after birth (Matzuk et al., 1995). Though no gonadal abnormalities were reported, follistatin clearly plays important regulatory roles in postnatal ovaries and testes (reviewed in (de Kretser et al., 2001; Knight and Glister, 2001)). I believe it may be worth reexamining follistatin null embryonic ovaries for expression defects using the somatic and germ cell markers of ovarian differentiation that have recently become available.

The second ovarian gene, *Adamts19*, is a new member of the ADAMTS family of secreted metalloproteases. Other members of this family are implicated in a range of processes including inflammatory response, angiogenesis, and organogenesis (Tang,

2001). At least one mammalian family member, *Adamts1*, is required for female fertility and appears to be involved in ovulation (Espey et al., 2000; Shindo et al., 2000). In addition, gonad formation in *C.elegans* is disrupted when the ADAMTS encoding gene *Gon-1* is mutated (Blelloch and Kimble, 1999). Thus, the expression of *Adamts19* in embryonic and postnatal ovaries makes it an exciting candidate for functional analysis.

Secreted Factors of the Testis

The Sertoli cells of the embryonic testis are thought to direct the organization of the bipotential gonad into a testis. Based on the differentiation and partitioning of cell types that occurs during this organization and the extensive cell migration into the testis, one would predict that multiple signals are generated by the Sertoli cells and by the responding cell types. Several of the testis genes that I identified encode secreted proteins (*Spp1*, *Cbln1*, *Cbln4*, *Tet2*, and *Tet21*) and may participate in some of these signaling events. Most of the genes encoding secreted proteins that were previously known to exhibit a sexually dimorphic expression pattern (*Mis*, *Dhh*, *vanin-1*, *protease nexin-1*, and *testatin*) are produced by Sertoli cells. The one exception that was recently reported is *renin*, which is produced by interstitial cells of the testis (Fig 2S and (Perera et al., 2001)). I have found that both *Tet21* and *Cbln1* are also expressed in interstitial cell types (Fig. 2 O and R).

Tet21 shares homology with the recently identified *sclerostin* gene (*SOST)* (Balemans et al., 2001; Brunkow et al., 2001). *Tet21* and *SOST* encode proteins that are ~39% identical and both contain a secretion signal and a cysteine-knot motif, characteristics of many signaling molecules (Vitt et al., 2001). In humans, mutations in *SOST* result in progressive bone overgrowth and it is postulated that SOST is involved in

the regulation of bone homeostasis. The second interstitially expressed factor, CBLN1 is a member of the cerebellin family of secreted neural peptides that share homology with the C-terminal globular domain of the C1q complement subunits (Urade et al., 1991). The cerebellins are capable of forming homo- and heteromeric complexes (Pang et al., 2000), and protease processing of CBLN1 results in the formation of a hexadecapeptide. Although the *in vivo* roles of the different protein products of the *Cbln1* gene are not known, the hexadecapeptide is present in both the cerebellum and the adrenal gland and is capable of inducing steroid secretion in adrenal gland slices *in vitro* (Albertin et al., 2000; Mazzocchi et al., 1999). *Tet21*, *Cbln1*, and *renin* all show different patterns of expression indicating that these transcripts are produced by different subsets of cells (Fig 2 O, R, and S). In the adult testis, the primary interstitial cell type is the steroid producing Leydig cell. However, the origins of the Leydig cell and its differentiation during embryonic development are ill defined (Habert et al., 2001; Merchant-Larios and Moreno-Mendoza, 1998). The study of *Tet21*, *Cbln1*, *renin*, and other markers of interstitial cells may help to determine the number of distinct interstitial cell types in the embryonic testis, their origins, and their roles in testis organogenesis.

The remaining testis secreted factors that I identified (Osteopontin, TET2, and CBLN4) are all produced within the testicular cords, most likely from the Sertoli cells (Fig 2E, G, H and Fig. 5 A and B). Osteopontin is a secreted glycoprotein that is capable of interacting with integrins and that has been implicated in numerous functions including bone mineralization and inflammatory response (Denhardt et al., 2001; Giachelli and Steitz, 2000). However, osteopontin null mice are viable and fertile with no reported reproductive phenotype (Liaw et al., 1998). CBLN4 is a new member of the cerebellin

family of proteins discussed above and is 77% identical to CBLN1 at the amino acid level (excluding the N-terminal secretion signal). Despite their similarity, *Cbln1* and *Cbln4* are conspicuously different in their pattern of expression. Finally, TET2 is a small novel protein that has limited homology to the β-defensins. The β-defensins have antimicrobial activity and are believed to be part of the innate immune response (Lehrer and Ganz, 2002). A role in epididymal function and sperm maturation has been proposed for another β-defensin-like molecule, BIN1b (Li et al., 2001).

Other testis genes

In addition to transcripts that encode secreted proteins, I identified a variety of other genes. For several of these genes there is functional data already available. α-Dystrobrevin (DTNA) is a cytoplasmic protein that interacts with and has homology to dystrophin, mutation of which results in Duchenne and Becker muscular dystrophies. These proteins together with other components form the dystrophin-containing glycoprotein complex (DGC) that links laminin α2 in the basal lamina to the cytoskeleton of the muscle fiber. Homozygous null mice for *Dtna* have mild skeletal and cardiac myopathies, but are viable and fertile (Grady et al., 1999). It has been proposed that a homologous protein, β-dystrobrevin, may partially compensate for the loss of Dtna.

Functional information is also available for *Pak3*. The p21-activating kinases (PAKs) including Pak3 were originally identified based on their ability to bind G proteins involved in the control of actin cytoskeletal organization (Manser et al., 1995). A truncation of the human PAK3 protein results in loss of kinase activity and causes nonsyndromic X-linked mental retardation (Allen et al., 1998). As with *osteopontin* and *Dtna*, no gonadal phenotype is known to be associated with this mutation.

Hedgehog-interacting protein (HHIP) is a membrane bound glycoprotein that is thought to attenuate hedgehog signaling by binding to hedgehog proteins (Chuang and McMahon, 1999). *Desert hedgehog* (*Dhh*) has previously been shown to have a male specific function. *Dhh* is upregulated in XY gonads by E11.5 and is expressed in Sertoli cells. Targeted deletion of *Dhh* results in spermatogenic defects and male infertility (Bitgood et al., 1996). Since *Hhip* has been shown to be upregulated in cells that are adjacent to hedgehog expressing cells, it is not surprising that I find that *Hhip* is expressed in interstitial cells of the embryonic testis. Patched, another hedgehog binding protein, is also upregulated in response to hedgehogs and is expressed in interstitial cells of embryonic and adult testes.

The final gene for which some functional characterization has been performed is *Cyp26b1*. The human ortholog of Cyp26b1 has been shown to act as a retinoic acid hydroxylase and is capable of hydroxylating all-trans-retinoic acid (White et al., 2000). Unlike all-trans-retinoic acid, the hydroxylated metabolites produced by Cyp26b1 can not activate any of the known retinoic acid receptors. Thus, it is believed that Cyp26b1 regulates retinoic acid signaling by inactivating all-trans-retinoic acid. A role for retinoids in the post-natal testis has been clearly established (Kastner et al., 1996; Lufkin et al., 1993; van Pelt and de Rooij, 1991), and the presence of various RAR and RXR retinoid receptors in embryonic testes and ovaries has been documented (Dufour and Kim, 1999; Morita and Tilly, 1999). Although a role for retinoic acid in embryonic gonads has not been demonstrated *in vivo*, exposure of embryonic testes to retinoic acid in organ culture disrupts cord formation (Livera et al., 2000; Livera et al., 2001). Similar experiments with embryonic ovaries indicate that exposure of retinoic acid promotes germ cell

survival, proliferation, and meiotic entry (Livera et al., 2000; Morita and Tilly, 1999). My identification of a differentially expressed regulator of retinoic acid activity strengthens the case for the involvement of retinoids in gonadal development.

Subtraction Assessment

My testis subtraction succeeded in recovering the majority of previously identified genes that are upregulated in embryonic testis relative to embryonic ovary as well as fourteen new genes. Likewise, my ovary subtraction was successful in identifying two new differentially expressed genes. Despite my successful isolation of new genes, it seems apparent that I identified only a small fraction of the differences that are present. My subtraction had a built in bias for abundant differentially expressed transcripts that show large expression differences. These are the easiest genes to identify by subtraction and other expression based approaches. Additional expression differences including transcripts that are less abundant or that have expression differences of smaller magnitudes are certain to exist. Complementary approaches being pursued by others will undoubtedly uncover many of these differences (Bowles et al., 2000; Grimmond et al., 2000; Perera et al., 2001; Tohonen et al., 1998).

It is my expectation that the identification of these genes will deepen our understanding of gonadal sex differentiation by providing insights into different complexes and pathways that are present in differentiating gonads, by serving as markers of different gonadal cell types, and ultimately through the functional analysis of candidate genes.

Materials and Methods

Mice and Tissues

Male and female C57BL/6 mice from Taconic Farms Inc. (Germantown, NY) were used for all experiments except for those using *W^v/W^v* mutant mice. *W^v/W^v* mice were produced through heterozygous matings. Timed matings were performed with the day a vaginal plug was found designated as E0.5. Germ cell depleted gonads were generated by injecting E9.5 pregnant females with 0.2 ml of 6.6 mg/ml busulfan (Sigma) in 50% DMSO (53mg/Kg body weight) (Merchant, 1975). Gonads that were isolated for total RNA preparation were dissected out and the adjacent mesonephros was removed. Gonads for whole-mount *in situ* hybridization were dissected out in PBS and fixed overnight at 4°C in 4% paraformaldehyde. Tissues were subsequently washed and stored at -20°C in methanol until used.

cDNA Subtraction

I performed a PCR based cDNA subtraction based on previously reported protocols with substantial modifications (Diatchenko et al., 1996; Lavery et al., 1997; Wang and Brown, 1991). Total RNA was isolated separately from E12.5 testes and E12.5 ovaries with Trizol (GIBCO-BRL), and mRNA was subsequently purified using the Micro-FastTrack mRNA isolation kit (Invitrogen). Double-stranded cDNA was generated with 100ng of mRNA and the TimeSaver cDNA synthesis kit (Amersham Pharmacia). PCR-amplifiable cDNA pools from testis and ovary were generated as previously reported (Wang and Brown, 1991). In brief, double-stranded cDNA was digested with AluI or AluI and RsaI, and adapter sequences were ligated onto the resulting cDNA fragments. Two testis cDNA pools were generated, each ligated to a different adapter (testis pool "A" adapter sequence: 5'-TAGTCCGAATTCAAGCAAGAG-3'; testis pool "B" adapter sequence: 5'-ATGCTGGATATCTTGGTACTC-3') and two ovary cDNA pools were generated, each ligated to a different adapter (ovary pool "F" adapter

sequence: 5'- CCACCATCTAGACATCGG-3'; ovary pool "G" adapter sequence: 5'-GTATGTCAACCGTCGACG-3').

Biotinylated testis pool "A" cDNA was generated by incorporating biotinylated dATP (GIBCO-BRL) by PCR amplification. The first round of subtraction was carried out by combining 200 ng of biotinylated testis pool "A" cDNA with 200 ng of PCR amplified testis pool "B" cDNA and 10 μg of amplified ovary cDNA. The mixed cDNA was ethanol precipitated, air dried, and resuspended in 2 μl of hybridization buffer (Lavery et al., 1997). This mixture was heated under oil to 95°C for 10 minutes and slowly cooled to 68°C over a 45 minute period. After two days at 68°C, biotinylated DNA fragments were recovered with streptavidin magnetic beads (Roche). This cDNA was reamplified using a primer specific for the testis pool "B" adapter. This pool is enriched for testis specific cDNAs and was designated testis pool "B_1". A second identical testis subtraction was performed with the exception that the testis pool "B" was biotinylated and pool "A" was not, and the final amplification was with a primer specific for adapter "A." These enriched fragments were designated testis pool "A_1". The enriched testis pools "A_1" and "B_1" were then used to perform a second round of subtraction. This second round was carried out as above, with the exception that the amount of testis cDNA was decreased to 100 ng for both the "A_1" and "B_1" pools (ovary cDNA was kept at 10 μg). Third and fourth rounds of subtraction were performed with 10 ng and 1 ng of each testis pool, respectively. The reciprocal ovary subtraction was performed as above.

cDNA sequencing and analysis

The subtracted testis and ovary cDNA pools were cloned into the pAMP10 plasmid (GIBCO-BRL) and clones were randomly selected for sequencing. The resulting sequences were organized into contigs using the DNA alignment program Sequencher (Gene Codes Corp) and these contigs were compared against the NCBI databases.

RT-PCR

Total RNA was isolated from male and female genital ridges using Trizol (GIBCO-BRL) as directed by the manufacturer. 1 μg of total RNA was reverse transcribed with oligo $d(T)_{18}N$ using Superscript II (GIBCO-BRL) in a total reaction volume of 25 μl. PCR was performed using 0.5 μl of RT as template in a total volume of 20 μl (10 mM Tris-HCl (pH 9), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 0.2 mM dNTPS, 0.5 μM primers, 0.5 U Taq polymerase). PCR cycling conditions for all primers were as follows: 94°C (30 seconds), 60°C (30 seconds), 72°C (1 minute) for 25-30 cycles.

cDNA cloning

Full length cDNA sequences were derived from a combination of subtracted cDNA clones, conventional cDNA libraries, and 5' and 3' RACE. Three cDNA libraries derived from adult mouse testis were used (Clontech, Stratagene, and one library of our own construction) and one library generated from a mixture of E12.5 testes and ovaries (of our own construction).

Whole-mount *in situ* hybridization

Whole-mount *in situ* hybridizations were performed essentially as previously described (Wilkinson and Nieto, 1993). Digoxigenin labeled riboprobes were generated using cDNA fragments cloned into the TA cloning vector pCR2.1-TOPO or pCR4-TOPO (Invitrogen). Plasmids were linearized by restriction digestion and transcribed with T3 or T7 RNA polymerase in the presence of Dig-labeling mix (Roche).

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Chapter 3

The Onset of Sexually Dimorphic Gene Expression in XX and XY Genital Ridges of the Mouse Embryo

Douglas B. Menke and David C. Page

Abstract

The process by which different testicular and ovarian cell lineages differentiate has not been well characterized. I have investigated the initiation of sexually dimorphic gene expression in the genital ridge to examine the relationship between ovarian and testicular differentiation in the mouse. I have found that molecular markers of testis differentiation are first upregulated in central regions of the XY genital ridge, and that this expression subsequently spreads to the anterior and posterior poles of the gonad. This center-to-pole pattern is observed with genes that are expressed in Sertoli and interstitial cell lineages and with genes that are initially expressed equally in XX and XY gonads prior to sex differentiation. In a related observation, I have found that *follistatin*, a molecular indicator of ovarian differentiation, is upregulated in the XX genital ridge in a similar center-to-pole pattern. In addition, *follistatin* is initially upregulated at the anterior and posterior poles of XY genital ridges before being down regulated in a pattern that is complementary to the upregulation of testis genes. The examination of a second ovarian gene, *Adamts19*, has revealed that its expression is upregulated at the anterior pole of XX and XY genital ridges. This anterior expression subsequently spreads posteriorly in XX gonads and is eliminated in XY gonads. I conclude that Sertoli cells and interstitial cell types differentiate in central regions of XY gonads before differentiating in polar regions, and ovarian differentiation is initiated at the anterior and posterior poles of normal XY gonads before being shut down by the induction of testicular differentiation pathways. Furthermore, some ovarian lineages differentiate in a center-to-pole pattern and others in an anterior-to-posterior pattern.

Introduction

The differentiation of embryonic gonads as male is dependent upon the expression of *Sry*, the Y-linked testis-determining gene (Koopman et al., 1991). Though the expression of *Sry* in the genital ridges of mice was first reported over a decade ago, the mechanism by which this gene transforms the sexually undifferentiated (bipotential) gonad into a testis has remained elusive (Koopman et al., 1990). Multiple lines of evidence now indicate that *Sry* is expressed in a subset of somatic cells that are induced to differentiate as Sertoli cells (Albrecht and Eicher, 2001; Burgoyne et al., 1988). The Sertoli cells then recruit additional testicular cell types.

During testicular differentiation the Sertoli cells surround and enclose the germ cells as the testicular cords form. The Sertoli cells serve as supporting cells that are required for germ cell growth, differentiation, and survival. It has been proposed that granulosa cells, the ovarian cells that surround and support the oocytes, and Sertoli cells share a common precursor in the bipotential gonad (Burgoyne et al., 1988). In this model the expression of *Sry* in the common precursor would prevent granulosa differentiation and activate Sertoli cell differentiation. This model has gained significant support by recent work reported by Albrecht and Eicher of the creation of an *EGFP* transgene that is driven by the *Sry* promoter (Albrecht and Eicher, 2001). In *Sry-EGFP* mice, expression of the transgene in XY genital ridges accurately recapitulates the endogenous expression of *Sry* that has been observed by whole-mount *in situ* hybridization (Bullejos and Koopman, 2001). This expression occurs in a "center-to-pole" pattern in which expression is initiated in central regions of the genital ridge and then spreads to the anterior and posterior poles (Albrecht and Eicher, 2001). In XX genital ridges of *Sry-EGFP* mice, this center-to-pole expression pattern is also observed. Since this transgene recapitulates the pattern of *Sry* expression without causing sex-reversal, it afforded a unique opportunity to monitor the female fate of XX cells that express this transgene. Albrecht and Eicher found that cells that had the morphological characteristics of ovarian

granulosa cells expressed the transgene later in ovarian embryonic development. However, the lack of known markers of embryonic granulosa cells prevented them from conclusively identifying these cells.

The center-to-pole expression of *Sry* has also suggested a possible explanation for patterns of sex-reversal observed in different mouse models of XY sex-reversal (Albrecht and Eicher, 2001; Bullejos and Koopman, 2001). When mouse XY gonads are only partially sex-reversed, they generally contain testicular tissue in central regions and ovarian tissue at the poles (Eicher et al., 1982; Washburn and Eicher, 1983). Reduced or mistimed *Sry* expression could result in the initiation of testicular development in central regions put not at the poles (Albrecht and Eicher, 1997; Nagamine et al., 1999).

Based on the above observations, one would expect genes that are upregulated during Sertoli cell differentiation to be expressed in a center-to-pole pattern. In addition, non-Sertoli testicular lineages should presumably also exhibit this center-to-pole expression wave since they are induced to differentiate by Sertoli cells. I have recently identified multiple new genes that are specifically expressed in different cell types of the testis as well as new gene markers of ovarian development. I have used these markers to examine the onset of sexually dimorphic gene expression in different testicular and ovarian cell lineages in XX and XY genital ridges and to examine the differentiation state of feminized and masculinized regions of fully and partially sex-reversed XY gonads.

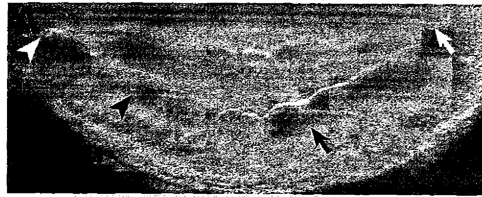
Results

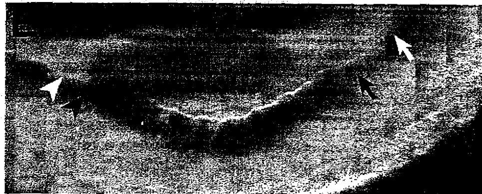
The onset of testis gene expression

The available evidence suggests that *Sry* expression induces the differentiation of the testicular Sertoli cells (Albrecht and Eicher, 2001; Burgoyne et al., 1988). By whole-mount *in situ* hybridization, *Sry* is detectable from ~14-24ts, where "ts" refers to the number of tail somites behind the hind limb bud (according to this method of staging embryos, eight tail somites (8ts) corresponds to ~E10.5, 18ts to ~E11.5, and 30ts to ~E12.5 (Hacker et al., 1995)). Expression of *Sry* is first observed in central regions of the gonad and then spreads to anterior and posterior regions (Bullejos and Koopman, 2001). It would logically follow that molecular markers of Sertoli cell differentiation should recapitulate this center-to-pole expression pattern with a slight delay since *Sry* induces Sertoli cell fate. I tested this hypothesis by examining the expression of *Tet4*, a presumptive marker of Sertoli cells that is abundantly expressed in the embryonic testis (*see Chapter 2*).

Prior to 17ts I detected no expression of *Tet4* in XX or XY genital ridges (data not shown). I first observed staining of *Tet4* in XY genital ridges at 17-18ts. The signal originated from small clusters of intensely staining cells located in central regions of the genital ridge (Fig. 1A). The zone in which *Tet4* expression appeared was offset more towards the anterior region of the genital ridge than the posterior. By 18-19ts *Tet4* was expressed in a larger number of cells in central locations and had spread outward towards the anterior and posterior tips of the genital ridge (Fig. 1C). This spreading of expression towards the anterior and posterior poles had extended further at 19-21ts (Fig. 1E). In the genital ridges that I examined, expression reached the anterior most tip by ~22ts and the posterior most tip ~24ts (Fig. 1G and I). I never observed *Tet4* expression in female genital ridges (Fig. 1B, D, F, H, and J). Thus, the onset of *Tet4* expression occurs in a center-to-pole pattern similar to that of *Sry*.

Figure 1. Whole-mount *in situ* hybridizations of *Tet4* on XY and XX embryonic gonads aged 17-24ts. Expression of the testis gene *Tet4* is initiated in central regions of XY gonads and spreads anteriorly and posteriorly A), C), E), G), and I). No expression is observed in XX genital ridges B), D), F), H), and J). All genital ridges are positioned with the anterior on the left and posterior on the right side of each panel. The ventral surface of the gonad is faced towards the top of each panel. White arrowheads on the left side of each panel indicate the anterior tip of the gonad, white arrows on the right indicate the posterior tip of the gonad, black arrowheads demarcate the anterior most expression, black arrows demarcate the posterior most expression.





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17-18 ts
18-19 ts
19-21 ts
22-23 ts
24 ts

Since Sertoli cells are responsible for masculinizing additional testicular cell types I decided to investigate the differential expression of *Cbln1*, a gene that is expressed in interstitial cells of the embryonic testis (*see Chapter* 2). In contrast to *Tet4*, *Cbln1* was detected in both XX and XY genital ridges from 13 to 16ts (Fig. 2A and B). By 19ts expression of *Cbln1* was upregulated in central and anterior regions of XY gonads but was absent in posterior regions (Fig. 2C). At this stage XX genital ridges had lost expression in central regions, but consistently retained expression at the very anterior tip (Fig. 2 D). Posterior staining was absent in most XX genital ridges at 19-21ts. However, I did detect faint expression at the posterior most tip of one XX genital ridge (Fig. 2 D). At 23-24ts expression in XY gonads had intensified and spread posteriorly, and expression in the anterior most regions of XX gonads was reduced (Fig.2 E and F). I also noted that the expression of *Cbln1* was confined to ventral regions of the genital ridges. In comparison, *Tet4* was expressed in inwardly located cells (compare Fig. 1E and G with Fig. 2C and E)

I extended my analysis to include three additional testis genes. Two of these, *Tet39* and *Cbln4*, are expressed in the testicular cords at E12.5, while the third gene, *Tet11*, is expressed in interstitial cells (*See Chapter* 2). I examined the expression of these genes at 20-22ts. All three of these genes were expressed predominantly in central and anterior regions of XY genital ridges (Fig. 3A, C, and E). Interestingly, *Tet11* was expressed in ventral regions as I previously observed for *Cbln1*, while *Tet39* and *Cbln4* were found in inner regions of the genital ridges. *Tet11*, *Tet39*, and *Cbln4* were not detected in XX genital ridges during this time interval (Fig. 3B, D, and F). I therefore conclude that the sexual differentiation of testicular cell lineages that express these genes occurs in a center-to-pole fashion.

The onset of ovarian gene expression

The down regulation of *Cbln1* in XX genital ridges occurs in a pattern that is complementary to that observed for the upregulation of testis genes. I examined the

Figure 2. Whole-mount *in situ* hybridization of *Cbln1* on XY and XX embryonic gonads aged 13-24ts. *Cbln1* is initially expressed in XY (A) and XX (B) genital ridges. Expression is upregulated in central regions of XY gonads and then spreads toward the anterior and posterior poles (C) and (E). Expression in XX gonads is lost in central regions, but is initially retained at the anterior and posterior poles (D) and (F). All genital ridges are positioned with the anterior on the left and posterior on the right side of each panel. The ventral surface of the gonad is faced towards the top of each panel. White arrowheads on the left side of each panel indicate the anterior tip of the gonad, white arrows on the right indicate the posterior tip of the gonad, black arrowheads demarcate the anterior most expression, black arrows demarcate the posterior most expression. In cases where expression is confined to the poles of the gonad, these expression domains are flanked with black arrowheads (anterior) or black arrows (posterior).

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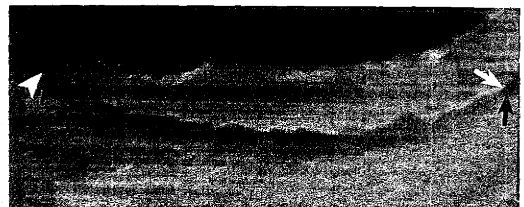
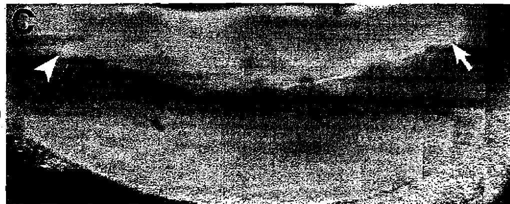
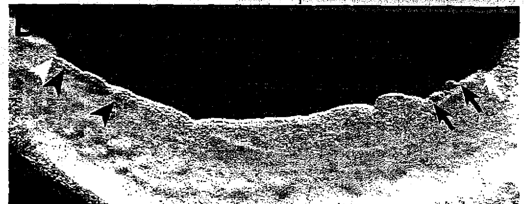
13-16 ts



19-21 ts





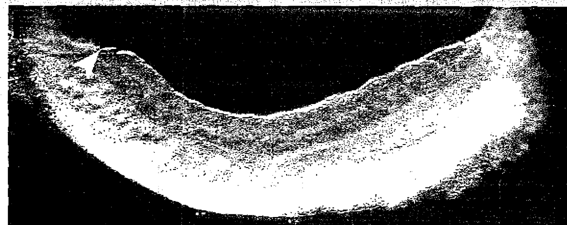
23-24 ts

Figure 3. Whole-mount *in situ* hybridization of *Tet11*, *Tet39*, and *Cbln4* in XY and XX embryonic gonads from 20-22ts. *Tet11* (A), *Tet39* (C), and *Cbln4* (E), are expressed in central regions of XY gonads at this developmental stage. Expression of these genes is not detected in XX gonads (B), (D), and (F). All genital ridges are positioned with the anterior on the left and posterior on the right side of each panel. The ventral surface of the gonad is faced towards the top of each panel. White arrowheads on the left side of each panel indicate the anterior tip of the gonad, white arrows on the right indicate the posterior tip of the gonad, black arrowheads demarcate the anterior most expression, black arrows demarcate the posterior most expression.

♂ ♀

B

C



expression of two gene markers of ovarian differentiation, *follistatin* and *Adamts19* (*see Chapter 2*), to determine whether the onset of sexual differentiation of XX genital ridges occurs in a similar center-to-pole pattern that is observed in XY gonads. *Follistatin* was undetectable in genital ridges at 13ts. However, by 16ts I easily detected expression in XX and XY genital ridges (Fig. 4A and B). *Follistatin* positive cells were scattered throughout most of the gonad, but the anterior and posterior tips were devoid of signal. At 18-19ts sexually dimorphic expression of *follistatin* was quite apparent (Fig. 4C and D). *Follistatin* expression had increased throughout the majority of the XX genital ridge, though the anterior and posterior most regions still lacked expression. At later stages *follistatin* expression had spread to these areas as well (Fig. 4H and J). In contrast to XX genital ridges, XY gonads displayed reduced *follistatin* expression at 18-19ts (Fig. 4C). Remarkably, by 20-21ts prominent *follistatin* expression was evident in anterior and posterior regions of XY genital ridges and was completely absent in central regions (Fig. 4E). Anterior expression was lost in XY gonads by 22-23ts, but expression at the posterior pole was observed as late as 24ts (Fig. 4G and I). I noted that the loss of *follistatin* expression in the posterior was often delayed in the right XY genital ridge as compared to the left ridge at 21-24ts (8 of 11 embryos aged 21-24ts had a clear right bias, the remaining three had roughly equal staining; data not shown). Additional XY genital ridges need to be tested to be certain of this apparent bias. However, this may be significant given the observation that in mouse models of XY sex-reversal the right gonad has a greater tendency to be sex-reversed than the left (Eicher and Washburn, 1983). The down regulation of *follistatin* in XY gonads occurs in a center-to-pole pattern that is consistent with the differentiation of XY gonads as male in central regions first followed by anterior and posterior locations. The upregulation of *follistatin* in XX gonads is also consistent with a similar pattern of female differentiation.

The final gene that I investigated is *Adamts19*. Prior to 19ts I did not detect the presence of *Adamts19* in XX or XY genital ridges. I observed *Adamts19* expression

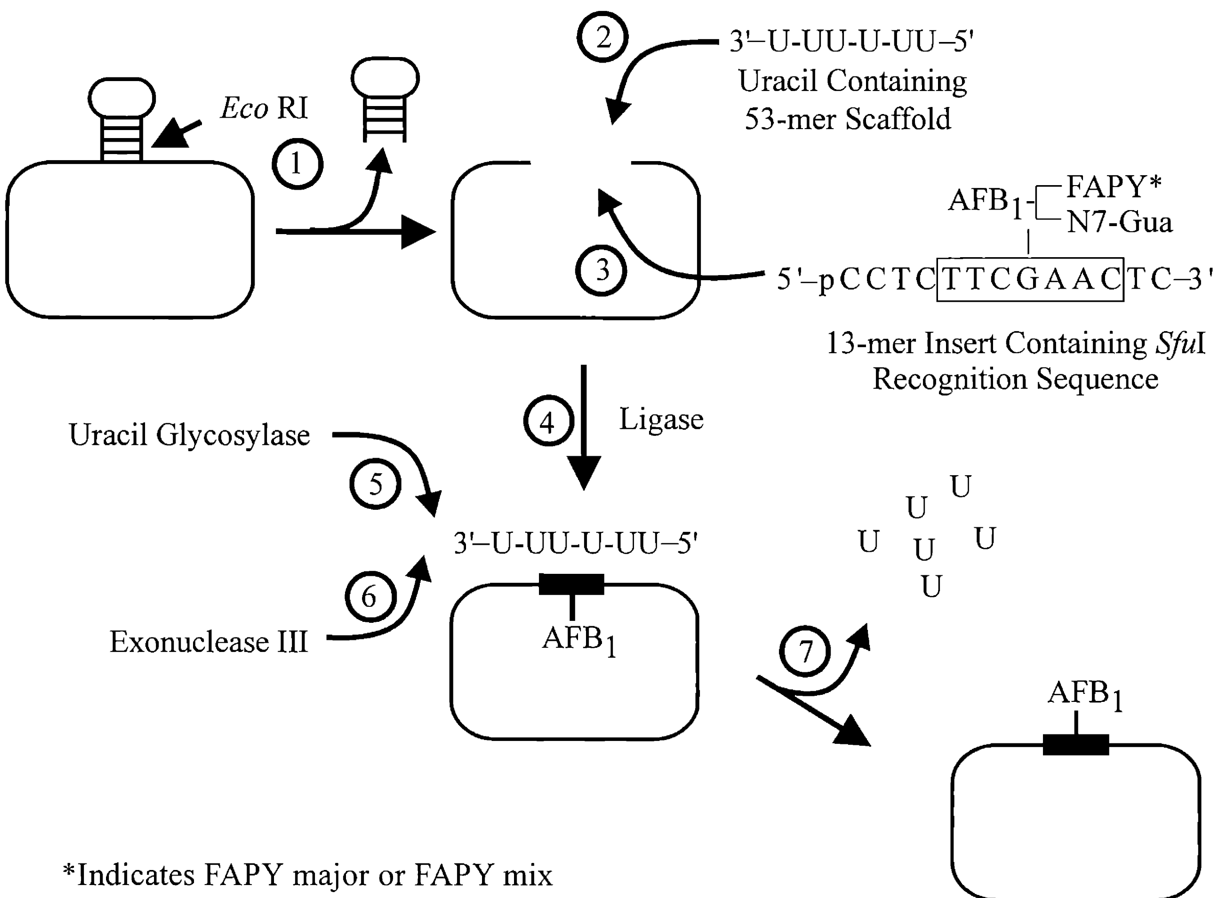


Figure 2.9: Genome construction. Single stranded M13mp7L2 viral genomes were cleaved with the *EcoR*I restriction enzyme (1) to yield a linear piece of DNA. This linear DNA was annealed to a 53 base uracil-containing oligonucleotide scaffold (2), which is complementary to 20 bases on either side of the excised region, forming a "gapped" structure. Either an unmodified or aflatoxin-containing 13 base oligonucleotide that was complementary to the unannealed 13 base region of the scaffold was then inserted into the gap (3). The oligonucleotide was then ligated (4). The scaffold was then removed using uracil glycosylase (5) and Exonuclease III (6), yielding a degraded scaffold (7) and an intact viral genome with a single modified guanine.

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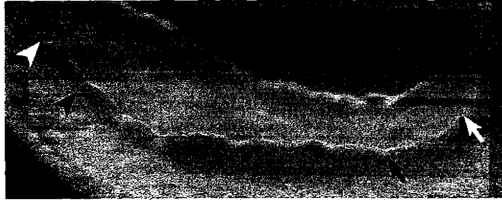
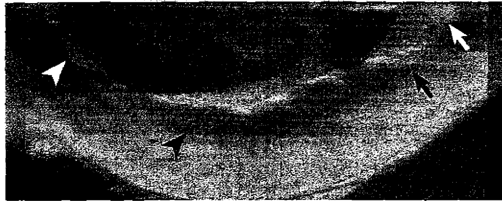
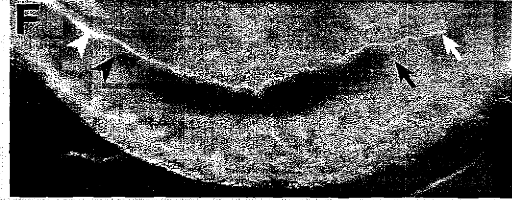
16-17 ts



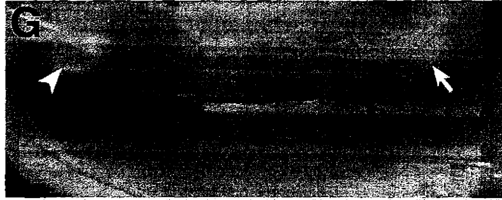
18-19 ts

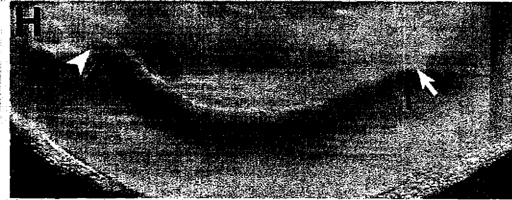


20-21 ts



22-23 ts





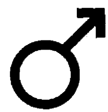
24 ts

at the anterior tips of XX and XY gonads beginning at ~19ts (Fig. 5A and B). By 24ts *Adamts19* expression had spread along the ventral aspect of XX genital ridges towards the posterior tip (Fig. 5D). At this stage staining in XY gonads was either absent or was confined to the anterior most portion of the gonad (Fig. 5C). From my previous work I know that this anterior expression in XY gonads is absent by E12.5 (*see Chapter 2*). The origin of *Adamts19* expression at the anterior tip of the genital ridge suggests that ovarian differentiation is not initiated entirely within central regions of the ovary.

Expression of testis and ovary genes in XY^{DOM} gonads

My examination of the onset of gene expression differences during gonadal sex differentiation revealed that the anterior and posterior regions of XY genital ridges initially upregulate ovarian genes. *Follistatin* is upregulated in anterior and posterior regions of XY genital ridges at 20-21ts (Fig. 4E) despite the fact that male differentiation has already been initiated in central regions of the XY gonad. Similarly, *Adamts19* is upregulated in the anterior most portions of XY genital ridges with the same timing that is observed in XX genital ridges (Fig. 5C and E). I made related observations with the down regulation of *Cbln1* expression in female gonads (Fig. 2D and E). I decided to explore this relationship by examining the expression of testis and ovary genes in sex-reversed XY^{DOM} gonads. Certain populations of *M. musculus domesticus* mice carry a Y chromosome, Y^{DOM}, that is not able to efficiently masculinize embryonic gonads when bred onto a C57BL/6 background (Eicher et al., 1982). These XY^{DOM} gonads often develop as ovotestes that contain ovarian tissue at the anterior and posterior poles and testicular tissue in the center. In some cases XY^{DOM} gonads develop as fully sex-reversed gonads. The likely cause of this sex reversal is insufficient expression of the *Sry* variant carried by the Y^{DOM} chromosome (Albrecht and Eicher, 1997; Nagamine et al., 1999). However, the reasons for this misregulation are not known.

Figure 5. Whole-mount *in situ* hybridization of *Adamts19* in XY and XX embryonic gonads from 19-24ts. *Adamts19* is first expressed at the anterior tip of XY (A) and XX (B) genital ridges. This anterior expression expands posteriorly in XX gonads (D), but not in XY gonads (C). All genital ridges are positioned with the anterior on the left and posterior on the right side of each panel. The ventral surface of the gonad is faced towards the top of each panel. White arrowheads on the left side of each panel indicate the anterior tip of the gonad, white arrows on the right indicate the posterior tip of the gonad, black arrowheads demarcate the anterior most expression, black arrows demarcate the posterior most expression. In cases where expression is confined to the poles of the gonad, these expression domains are flanked with black arrowheads (anterior) or black arrows (posterior).



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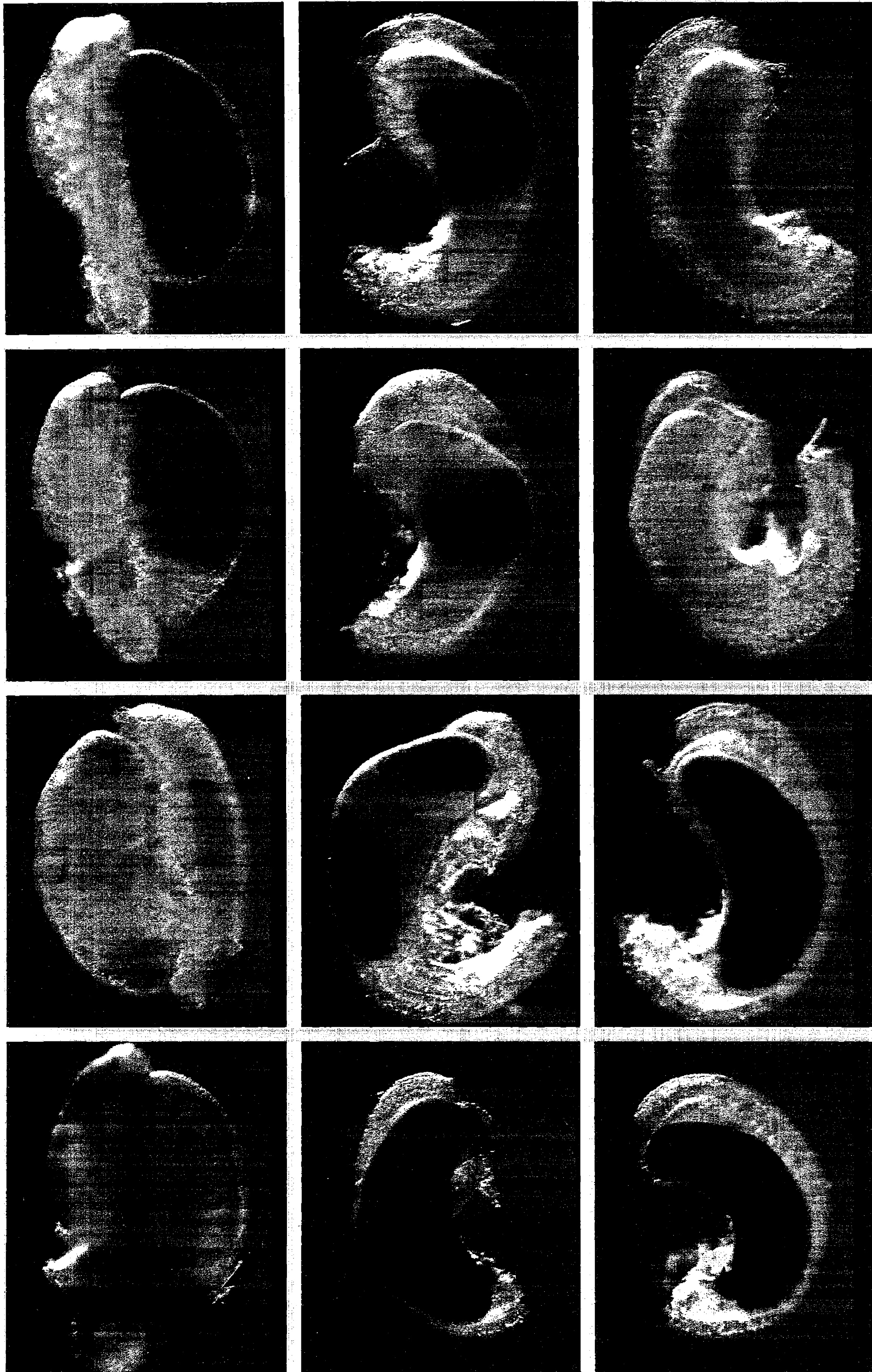
22-24 ts

I examined XY^{DOM} gonads at E14.5. At this developmental stage regions that are masculinized or feminized are easily identified by eye. In normal embryonic testes *Tet4* expression is limited to the testicular cords. As expected, *Tet4* expression was completely confined to masculinized regions that contained testicular cords in both mildly and severely effected XY^{DOM} gonads and was absent in fully sex-reversed gonads (Fig. 6A, B, and C). *Cbln1* expression was also confined to masculinized regions associated with testicular cords (Fig. 6D and E). As with *Tet4*, I observed no *Cbln1* staining in fully sex-reversed XY^{DOM} gonads (Fig. 6F). Rather than a gradual decline in expression, the boundary of *Cbln1* expression is very sharply defined and does not extend into regions that lack testicular cords.

In addition to testis genes, I also explored the expression of *follistatin* and *Adamts19* in sex-reversed gonads. Strong *follistatin* expression was detected in severely and fully sex-reversed XY^{DOM} gonads, and this expression was excluded from masculinized regions (Fig. 6H and I). However, when the feminized regions of the XY^{DOM} gonad were less extensive only faint *follistatin* expression was seen (Fig. 6G). *Adamts19* was also easily detected in severely and fully sex-reversed XY^{DOM} gonads (Fig. 6K and L). *Adamts19* staining was variable when the feminized region was small, sometimes demonstrating little expression and in other cases producing robust expression (Fig 6J).

Figure 6. Whole-mount in situ hybridization examining the expression of testis and ovary genes in XY^{DOM} gonads. *Tet4* expression in a very mildly feminized XY^{DOM} testis (A), a severely feminized XY^{DOM} ovotestis (B), and a completely sex-reversed XY^{DOM} ovary (C). Expression of Cbln1 in a mild XY^{DOM} ovotestis (D), a severely feminized XY^{DOM} ovotestis (E), and a completely sex-reversed XY^{DOM} ovary (F). Expression of *follistatin* in a mild XY^{DOM} ovotestis (G), a severely feminized XY^{DOM} ovotestis (H), and a completely sex-reversed XY^{DOM} ovary (I). Expression of *Adamts19* in a mild XY^{DOM} ovotestis (G), a severely feminized XY^{DOM} ovotestis (H), and a completely sex-reversed XY^{DOM} ovary (I). Gonads are positioned such that the anterior pole is at the top of each panel. Arrows in panels (G) and (J) demarcate the boundary between feminized and masculinized regions. Note in panel (J) intense *Adamts19* expression in feminized tissue at the posterior pole but not at the anterior pole.



Discussion

Differentiation of testicular cell lineages

I have examined the onset of sexually dimorphic gene expression patterns in XX and XY genital ridges during the earliest stages of gonadal differentiation. I have found that molecular markers of testis development are differentially expressed in central regions of embryonic XY genital ridges first. This domain of differential expression is located more anteriorly than posteriorly and subsequently spreads towards the anterior and posterior poles of the gonad. I found that this center-to-pole pattern of expression occurs with genes that are expressed in somatic cells of the testicular cords (*Tet4*, *Tet39*, and *Cbln4*) as well as with genes that are ultimately expressed in somatic cells that are located outside the cords (*Cbln1* and *Tet11*). In addition, this pattern is observed with genes that exhibit different expression states prior to the onset of sexually dimorphic expression. *Tet4* is initially expressed in neither XX nor XY genital ridges, whereas *Cbln1* is initially expressed in both sexes. Despite these differences I found that the upregulation of *Tet4* and *Cbln1* expression in XY gonads both occur in a center-to-pole pattern. In a complementary fashion, *Cbln1* expression is lost in XX genital ridges in central and posterior regions first and then at the anterior pole.

Sertoli cell differentiation is prompted by the expression of *Sry*, and it is the Sertoli cells that are believed to induce additional cells to differentiate into testicular cell types. Therefore, one might expect the differentiation of non-Sertoli cell types of the testis to appear first in regions of the XY gonad where Sertoli cell differentiation was initiated. My observation that *Cbln1* and *Tet11*, two gene markers of interstitial cell types, are upregulated in a spatial pattern similar that observed for *Sry* suggests that this is true for at least some interstitial cell types. In addition, these two genes both exhibit sexually dimorphic expression between 19-22ts, not long after Sertoli cell genes are first upregulated (reviewed in (Swain and Lovell-Badge, 1999)).

Labeling experiments have indicated that Sertoli cell precursors migrate into the gonad from the ceolomic epithelium that lies on the ventral surface of the genital ridge prior to 18ts (Karl and Capel, 1998; Schmahl et al., 2000). After this stage, the ceolomic epithelium continues to contribute to interstitial cell type populations but no longer contributes to the Sertoli cell population. The expression of *Sry* is first detected in inwardly located cells that lie below the ceolomic epithelium (Bullejos and Koopman, 2001). Therefore, genes that are upregulated in differentiating Sertoli cells should be expressed in inwardly located regions of the genital ridge and not in ventral regions around the ceolomic epithelium. *Tet4*, *Tet39*, and *Cbln4* meet these expectations. I also noted that both markers of interstitial cell types examined here, *Cbln1* and *Tet11*, are expressed along the ventral surface of the gonad (Compare Fig. 1I with Fig. 2E and compare Fig. 3A with 3E). This suggests to us that the lineage(s) that are characterized by *Cbln1* and *Tet11* expression may be derived from the ceolomic epithelium that lies along the surface of the gonad.

Ovarian Differentiation

The onset of sexually dimorphic expression of the ovarian genes that I investigated has provided additional insights into testis and ovary differentiation. The expression of *follistatin* in XX gonads occurs in a center-to-pole pattern, as I observed with the testis genes. Interestingly, *follistatin* is initially upregulated in both XY and XX genital ridges between 13 and 16ts. This upregulation occurs with roughly the same timing as *Sry*, which can first be detected by whole-mount *in situ* hybridization at 13 to 14ts (Bullejos and Koopman, 2001). The suppression of *follistatin* expression in the XY gonad appears to coincide fairly well with the timing of *Sry* expression. *Sry* is expressed first in central regions and is maximally expressed at ~18ts. It is at this time when I first observe the down regulation of *follistatin* in XY genital ridges. *Follistatin* is upregulated at the anterior and posterior tips of XY gonads by 20-21ts. Anterior expression is subsequently lost by ~22ts, and this is shortly followed by loss of posterior expression at

~24ts. Although *Sry* expression is lost in central regions of the gonad by 19ts, it is retained at the anterior and posterior poles until 20ts and 24ts, respectively. The correlation between *follistatin* and *Sry* expression suggests at least two possibilities. One possibility is that *Sry* and *follistatin* are both expressed in the Sertoli/granulosa cell lineage and that both of these genes are expressed in XY gonads as this cell lineage differentiates. Expression of *Sry* would then directly or indirectly extinguish the expression of granulosa genes, including *follistatin*. Since the expression of *follistatin* in granulosa cells of the postnatal ovary is well established, this appears to be a reasonable supposition (Shintani et al., 1997; Sidis et al., 1998). Alternatively, *follistatin* and *Sry* may be expressed in separate lineages, and the down regulation of *follistatin* could result from the masculinizing influence of differentiating Sertoli cells on the *follistatin* expressing lineage. These possibilities could be distinguished through colocalization studies using the recently reported *Sry-EGFP* transgenic mice (Albrecht and Eicher, 2001). In XX genital ridges follistatin should colocalize with EGFP positive cells if the *Sry* and *follistatin* expressing lineages are the same.

Adamts19 has an expression pattern that is very different from the other genes I have examined. Expression is initiated at the anterior tip of both XX and XY genital ridges and then spreads posteriorly in XX, but not XY, gonads. This anterior-to-posterior expression is significant because it suggests that ovarian differentiation is not initiated solely from central regions of the gonad. While it is possible that differentiation of central regions of the ovary may induce cells in the anterior to express *Adamts19*, the fact that differentiating XY genital ridges also initiate anterior *Adamts19* expression indicates that similar inductive signals would initially be present in XY gonads as well. It is currently unclear whether the spread of *Adamts19* expression is a result of the migration of a particular population of cells or a wave of differentiation. I also note that *Adamts19* is expressed by cells along the ventral surface of the ovary, in contrast to *follistatin*, which is expressed in cells located in inner regions. Anterior and ventral biases in expression are

also apparent for *Adamts19* in E12.5 and E13.5 ovaries (*see Chapter 2*). This suggests that *follistatin* and *Adamts19* are markers of two different ovarian cell populations. Since the absence of ovarian markers has hindered the study of ovarian cell lineages, I believe that *follistatin* and *Adamts19* will be quite useful in this regard.

The differentiation state of XY^{DOM} gonads

Partially sex-reversed XY^{DOM} ovotestes are typically masculinized in central regions and feminized at the anterior and posterior poles. In situations where only one pole is sex-reversed, it is more often the posterior pole that is feminized. The discovery that *Sry* exhibits a "center-to-pole" expression pattern coupled with the reduced level of *Sry* expression from the Y^{DOM} chromosome provides an apparent explanation for the position effects observed in XY^{DOM} ovotestes (Albrecht and Eicher, 2001; Bullejos and Koopman, 2001; Nagamine et al., 1999). My observation that ovarian genes are initially upregulated in anterior and posterior regions of XY genital ridges supports the idea that the poles of XY gonads begin to differentiate as female in normal XY gonads. It is therefore not surprising that reduced or late expression of *Sry* results in feminized regions at the poles. In addition to sex-reversal biases between central and polar regions, a left-right bias has also been noted in XY^{DOM} gonads (Eicher and Washburn, 1983). In XY^{DOM} embryos, the right gonad tends to be more severely sex-reversed than the left. It therefore may be significant that I found *follistatin* expression was often more extensive in the right genital ridge than of the left. A left-right bias has not been observed with *Sry* expression (Bullejos and Koopman, 2001). Thus, the underlying cause of the *follistatin* left-right bias in normal XY gonads may not be due to differences in the levels or timing of *Sry* expression.

There has been some question as to the differentiation state of feminized regions of ovotestes (Albrecht et al., 2000; Nagamine et al., 1987). These feminized regions contain meiotic oocytes. However, this is poor evidence that these regions contain fully differentiated ovarian tissue because outside of a testicular environment embryonic germ

cells will enter meiosis by default. My expression analysis of E14.5 XY^{DOM} gonads revealed that the domains of testis and ovary gene expression are very sharply demarcated. XY^{DOM} ovotestes that have extensive feminized regions strongly express two markers of ovarian differentiation. In ovotestes that have small feminized regions the expression of ovary genes is generally variable or reduced. Based on these results I conclude that severely effected XY^{DOM} ovotestes do have differentiated ovarian tissue. However, the feminized tissue in mildly sex-reversed XY^{DOM} ovotestes generally does not exhibit the strong expression of ovarian genes that is expected from fully differentiated ovarian tissue. Examination of XY^{DOM} gonads at earlier developmental stages will be required to determine whether the reduced expression of ovarian genes in mildly feminized XY^{DOM} is a result of early or late events.

Materials and Methods

Mice and Tissues

Male and female C57BL/6 mice from Taconic Farms Inc. (Germantown, NY) were used for all experiments. Timed matings were performed with the day a vaginal plug was found designated as E0.5. Gonads for whole-mount in situ hybridization were dissected out in PBS and fixed overnight at 4°C in 4% paraformaldehyde. Tissues were subsequently washed and stored at -20°C in methanol until used. Embryos were staged by counting the number of tail somites posterior to the hind limb bud as previously reported. Under this staging scheme eight tail somites (8ts) corresponds roughly to embryonic day 10.5 (E10.5), 18ts corresponds to ~E11.5, and 30ts corresponds to ~E12.5 (Hacker et al., 1995).

Whole-mount *in situ* hybridization

Whole-mount *in situ* hybridizations were performed essentially as previously described (Wilkinson and Nieto, 1993). Digoxigenin labeled riboprobes were generated using cDNA fragments cloned into the TA cloning vector pCR2.1-TOPO or pCR4-TOPO (Invitrogen). Plasmids were linearized by restriction digestion and transcribed with T3 or T7 RNA polymerase in the presence of Dig-labeling mix (Roche).

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Chapter 4

Sex Differentiation and Meiotic Entry of Female Germ Cells Occurs in an Anterior-Posterior Wave

Douglas B. Menke and David C. Page

Abstract

The differentiation of murine germ cells as male or female during embryogenesis is dependent upon the somatic environment in which they develop rather than their sex chromosome content. Sex differentiation of female germ cells is morphologically evident at embryonic day 13.5 (E13.5) as the first female germ cells enter meiosis. The results presented here demonstrate that the *Stra8* gene is a molecular marker of female germ cell sex differentiation. *Stra8* is upregulated in female germ cells prior to meiotic entry and is not expressed in male embryonic germ cells. Moreover, *Stra8* expression in partially and fully sex-reversed XY^{DOM} gonads occurs only in feminized regions. Therefore, the appearance of Stra8 in germ cells is correlated with the somatic environment and not the sex chromosome content of the germ cell. A developmental time course of *Stra8* expression has further revealed that sex differentiation of female germ cells takes place in an anterior-to-posterior wave that is initiated by embryonic day 12.5 (E12.5). Consistent with these results, I find that meiotic entry of female germ cells also occurs in an anterior-to-posterior wave. Thus, meiotic entry and female sex differentiation may be affected by local environmental cues that are present in the differentiating XX gonad.

Introduction

The events that underlie sex determination and differentiation of the mammalian gonad are critical for the generation of functional gametes in the adult animal. Recent progress has helped to elucidate some of the cellular and molecular events that transpire during differentiation of the testicular soma during embryogenesis (Albrecht and Eicher, 2001; Capel et al., 1999; Colvin et al., 2001). However, the fundamental mechanisms involved in male and female sex differentiation remain largely unexplained. This is particularly evident in regards to sex differentiation of the germ cell lineage.

Primordial germ cells of the mouse are first identifiable at approximately embryonic day 7.2 at the base of the allantois (Ginsburg et al., 1990). The germ cells of male and female mouse embryos are behaviorally and morphologically indistinguishable as they begin their migration to the gonads from their extraembryonic location at ~E8.5 and as they enter the bipotential gonad from E10-E11.5. The differentiation of the somatic elements of the testis and ovary is visually evident by E12.5, however, it is not until E13.5 that morphological differences between male and female germ cells become apparent (reviewed in (McLaren, 2000)). It is at this stage that the first meiotic female germ cells appear in the embryonic ovary. The entry of ovarian germ cells into meiotic prophase continues over the next two days with the majority of germ cells having entered meiosis by E15.5 (Peters, 1970; Peters et al., 1962). Over this same time period, testicular germ cells cease their mitotic proliferation and arrest as prospermatogonia (McLaren, 1984).

The differentiation of germ cells as male or female is dependent upon the developmental environment rather than the sex chromosome content of the germ cell.

This conclusion was initially based on observations of XX↔XY chimeric gonads and on different mouse models of sex reversal (McLaren, 1981; Palmer and Burgoyne, 1991; Taketo-Hosotani et al., 1989). These studies revealed that XX embryonic germ cells in a testicular environment will develop as male prospermatogonia and similarly, XY germ cells in ovarian surroundings will enter meiosis as oocytes. Moreover, the discovery that both XY and XX ectopic germ cells that mistakenly enter the adrenal gland develop as oocytes suggested that germ cells will differentiate as female unless masculinized by a testicular environment (Zamboni and Upadhyay, 1983).

Germ cells in the developing ovary enter meiotic prophase from E13.5 to E15.5. It is this embryonic initiation of meiosis that is considered definitive evidence for differentiation of germ cells as "female." Yet it is evident that germ cells commit to differentiation as female or male before this developmental stage. Germ cells isolated from XY gonads at E11.5 will enter meiosis and develop as oocytes if grown in lung reaggregates, in reconstituted ovaries, or in tissue culture (Adams and McLaren, 2002; Chuma and Nakatsuji, 2001; McLaren and Southee, 1997). However, by E12.5 the majority of XY germ cells will develop as male and will not enter meiosis if grown under these conditions. Likewise, XX germ cells isolated at E12.5 or earlier will mitotically arrest and develop as male prospermatogonia if placed in reconstituted testes, whereas E13.5 XX germ cells develop as oocytes under the same conditions. Hence, testicular germ cells commit to the male pathway by E12.5 and ovarian germ cells commit to the female pathway by E13.5. For this reason one would expect there to exist gene expression differences between germ cells developing in embryonic testes and ovaries by

E12.5. With this in mind I have examined the onset of changes in gene expression that become apparent as ovarian germ cells undergo female sex differentiation.

Results

Stra8 is expressed in female germ cells embryonically

In the course of performing experiments to identify genes that are differentially expressed between male and female embryonic gonads (paper in preparation), I discovered that *Stra8*, one of my "male" control genes, is expressed in female and not male embryonic gonads. The *Stra8* gene was originally identified in a cDNA subtraction designed to detect genes that are upregulated in P19 embryonal carcinoma cells in response to retinoic acid (Bouillet et al., 1995). *Stra8* is an autosomal gene located on mouse chromosome six and encodes a 393 amino acid protein of unknown function. *Stra8* was previously reported to be specifically expressed in germ cells of embryonic and adult male mice (Oulad-Abdelghani et al., 1996). Contrary to this previous report, my RT-PCR results demonstrate that *Stra8* is expressed in embryonic mouse ovaries from E12.5 to E14.5 (Fig. 1). To examine the expression of *Stra8* in greater detail, I performed whole-mount *in situ* hybridizations on E13.5 ovaries and testes (Fig. 2A). Cells strongly positive for Stra8 mRNA are present in E13.5 ovaries but are not detected in E13.5 testes. Notably, in E13.5 ovaries there are clearly more positive cells present in anterior regions than in posterior regions.

Since *Stra8* is expressed in spermatogonial germ cells of juvenile and adult mouse testes (Oulad-Abdelghani et al., 1996; Wang et al., 2001), I determined whether the *Stra8* positive cells in E13.5 ovaries are germ cells. Sectioning of my E13.5 whole-mounts revealed that *in situ* staining colocalized with cells containing large round nuclei, a distinctive feature of embryonic germ cells (Fig. 2B and C). These nuclei are easily distinguished from the smaller nuclei of the surrounding somatic cells. *Stra8* positive

Figure 1. Detection of sex specific Stra8 expression by RT-PCR. Expression of *Gapd* (ubiquitous control), *Mis* (testis specific control), and *Stra8* in E12.5, E13.5, and E14.5 testes and ovaries.

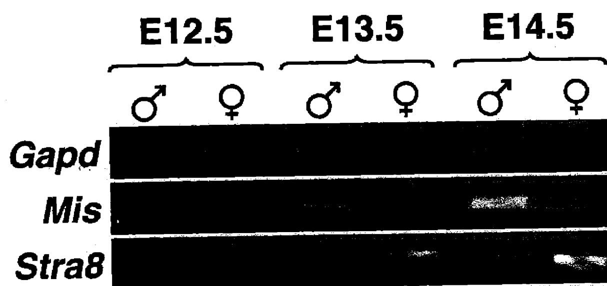
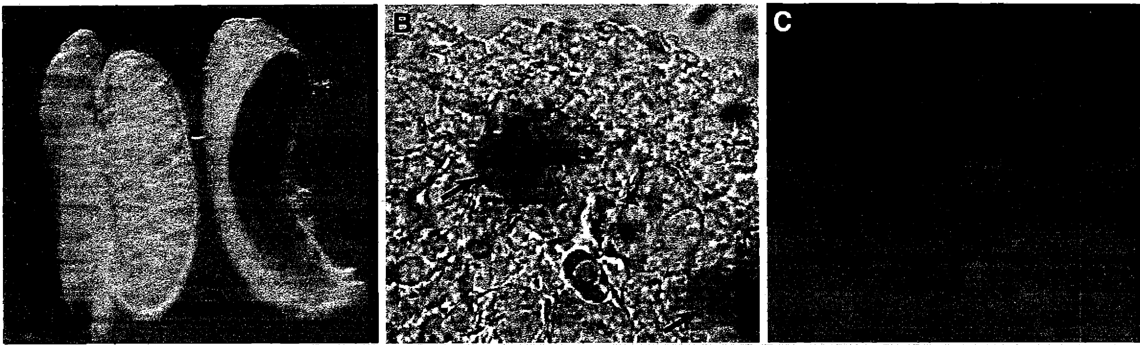
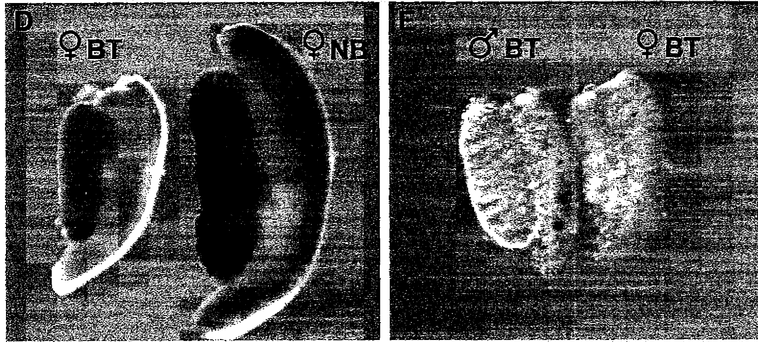

E12.5
E13.5
E14.5
♂
♀
♂
♀
♂
♀
Gapd
Mis
Stra8

Figure 2. Expression of *Stra8* in normal and germ cell depleted embryonic gonads. *Stra8* whole-mount *in situ* hybridizations on E13.5 gonads reveals *Stra8* expressing cells (stained purple) in ovaries but not testes (A). Paraffin sections of E13.5 ovarian whole-mounts were counterstained with DAPI and observed under bright-field (B) and fluorescent (C) illumination. *Stra8* expressing germ cells (arrows) are located next to a cluster of *Stra8* negative germ cells (arrowhead). Detection of all germ cells by staining for endogenous alkaline phosphatase activity demonstrates the absence of germ cells in busulfan treated ovaries and the even distribution of germ cells in normal untreated ovaries (D). No signal is observed in *Stra8* whole-mount *in situ* hybridizations on E13.5 busulfan treated testes and ovaries (E). BT = Busulfan Treated, NB = No Busulfan.


B
C


D
♀BT
♀NB
E
♂BT
♀BT

cells were found either as single isolated germ cells or in clusters of two to four cells. Clusters of *Stra8* negative germ cells were often seen in close proximity to *Stra8* expressing germ cells. I chemically treated embryos with busulfan to confirm the germ cell specific expression of *Stra8*. Busulfan treatment preferentially kills germ cells, and can be used to almost entirely deplete embryonic gonads of germ cells (Merchant, 1975). Detection of endogenous alkaline phosphatase activity in treated and untreated E13.5 ovaries demonstrated that almost all germ cells were eliminated with busulfan (Fig. 2D). Similarly, *Stra8* staining was completely absent in busulfan treated ovaries (Fig. 2E). In contrast, expression of the somatically expressed ovarian gene follistatin (paper in preparation) was not effected by busulfan (data not shown).

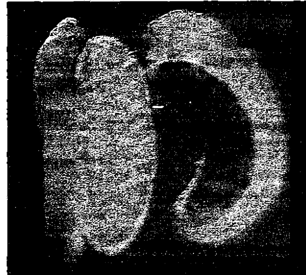
Sex differentiation of female germ cells occurs in an anterior-posterior wave

Visualization of the germ cells at E13.5 by AP staining revealed an even distribution of germ cells throughout the ovary (Fig. 2D). This stands in contrast to the distribution of *Stra8* positive germ cells observed in E13.5 *in situ* hybridizations in which I noted more positive cells in the anterior portion of the ovary (Fig. 2A). I therefore decided to determine the developmental stages at which *Stra8* positive cells are present in embryonic ovaries and to examine the distribution of these *Stra8* expressing cells along the anterior-posterior axis of the ovary (Fig. 3). I observed no *Stra8* staining in E11.5 and E12 XX and XY gonads (data not shown). I first detected strongly positive cells in E12.5 ovaries (Fig. 3A). These cells were located almost exclusively in the anterior half of the ovaries I examined. Consistent with my previous results, at E13.5 I noted a significant anterior bias (Fig. 3B), though not as

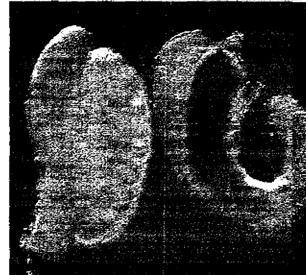
Figure 3. Developmental time course of *Stra8* and *Dmc1* expression. Whole-mount *in situ* hybridization for *Stra8* on testes and ovaries at E12.5 (A), E13.5 (B), E14.5 (C), E15.5 (D), and E16.5 (E). Whole-mount *in situ* hybridization for *Dmc1* on testes and ovaries at E12.5 (F), E13.5 (G), E14.5 (H), E15.5 (I), and E16.5 (J). In all panels testes are located on the left and ovaries on the right.

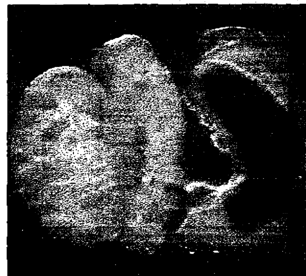
Stra8 | **Dmc1**

E12.5

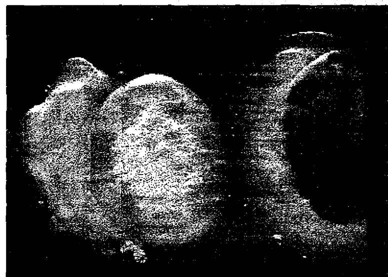


E13.5

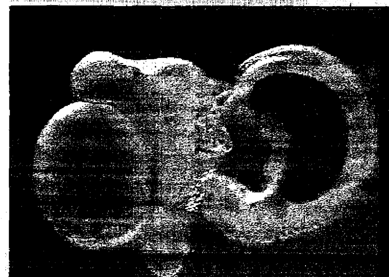




E14.5



E15.5



E16.5

dramatic as that seen at E12.5. At E14.5 *Stra8* staining was observed throughout the ovary (Fig. 3C). However, by E15.5 *Stra8* expressing cells were located predominantly in the posterior half of the ovary (Fig. 3D). Only a small number of *Stra8* positive cells were found at E16.5, primarily at the posterior most tip of the ovary (Fig. 3E). Thus *Stra8* is expressed in an anterior-to-posterior wave that lasts approximately four days. I observed no *Stra8* positive cells in embryonic testes.

The expression of *Stra8* in female embryonic germ cells and its absence in male embryonic germ cells implies that *Stra8* is a molecular indicator of female sex differentiation. Furthermore, the occurrence of *Stra8* expression in a wave that starts anteriorly suggests that female sex differentiation of germ cells also transpires in such a wave. Historically, embryonic entry into meiosis has been considered the defining hallmark of female germ cell sex determination, and the morphological changes characteristic of meiotic germ cells and the expression of meiotic genes have been used as a markers of germ cell sexual identity (Chuma and Nakatsuji, 2001; McLaren and Southee, 1997). I therefore predicted that if differentiation of ovarian germ cells occurs in a wave, meiotic genes should be first upregulated in germ cells located in the anterior portion of the ovary. I performed whole-mount *in situ* hybridizations for the meiotic specific *Dmc1* gene to test this hypothesis. *Dmc1* is a *RecA* homologue that is expressed during meiotic prophase and which is required for chromosome synapsis and progression through male and female meiotic prophase (Pittman et al., 1998; Yoshida et al., 1998). At E12.5 I detect faint staining for *Dmc1* in the anterior portion of ovaries (Fig. 3F). By E13.5 I see strong expression of *Dmc1* with a definite anterior bias (Fig. 3G). *Dmc1* positive cells are found uniformly throughout the ovary from E14.5 to E15.5 (Fig. 3H and

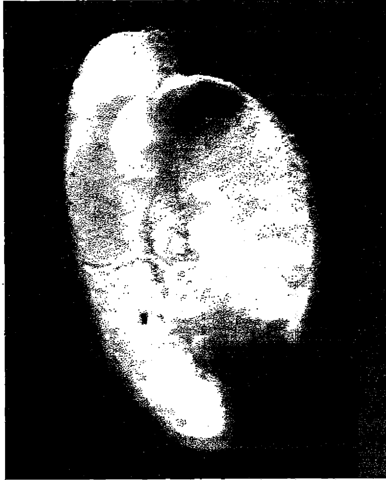
I). However, at E16.5 I observe a reduction of *Dmc1* in anterior regions (Fig. 3J). Thus, entry into meiosis as determined by expression of *Dmc1* generally occurs earlier in germ cells located in anterior regions of the ovary than in posterior regions.

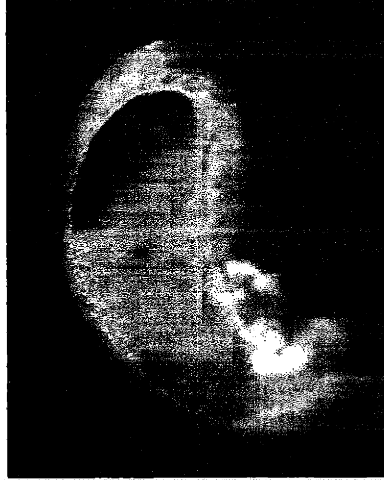
Stra8 is expressed in sex reversed XY^{DOM} embryonic gonads

If the expression of *Stra8* in embryonic germ cells is a molecular marker of female sexual differentiation, then one would expect *Stra8* to be expressed in germ cells that have differentiated as "female" regardless of their sex chromosome constitution. I obtained fully and partially feminized XY embryonic gonads by breeding male *M. musculus domesticus* mice that carry a Y^{DOM} chromosome to C57BL/6 females (see Materials and Methods). These male mice carry a Y chromosome (Y^{DOM}) that is not capable of properly masculinizing developing X Y^{DOM} gonads when bred onto a C57BL/6 background (Eicher and Washburn, 1983; Eicher et al., 1982). This effect is likely due to misexpression of the Y^{DOM} *Sry* allele (Albrecht and Eicher, 1997). These XY^{DOM} embryonic gonads often develop as ovotestes that contain regions of testicular tissue in central regions of the gonad and ovarian regions at the anterior and posterior poles. In some instances, XY^{DOM} gonads are completely feminized and form ovaries. Germ cells in feminized regions of XY^{DOM} ovotestes and ovaries initiate oogenesis (Taketo-Hosotani et al., 1989). Though XY^{DOM} oocytes are capable of being fertilized, most of the resulting embryos die at the 1-2 cell stage (Amleh et al., 1996; Merchant-Larios et al., 1994). In E14.5 XY^{DOM} ovotestes and ovaries I find *Stra8* expressing cells in regions of the gonad that are feminized (Fig. 4A, B, and C). In XY^{DOM} ovotestes *Stra8* positive cells are located at the poles and are not detected in regions that contain testicular cords (Fig. 4A, 4B, and 4D). In E14.5 XY^{DOM} ovaries I find *Stra8* expression throughout the gonad, just

as I observe with normal E14.5 XX ovaries (Compare Fig. 3C and Fig. 4C). I conclude that *Stra8* is a reliable indicator of female sexual differentiation.

Figure 4. Expression of Stra8 in feminized E14.5 XY^{DOM} gonads. Whole-mount in situ hybridization of *Stra8* on mildly sex-reversed ovotestis (A), moderately feminized ovotestis (B), and fully feminized XY^{DOM} ovary (C). Double whole-mount in situ for Stra8 and Tet4. on mildly sex reversed ovotestis (D). Stra8 expression (purple) is limited to regions that lack testicular cords as revealed by the testis gene Tet4 (orange-brown).





Discussion

I have established that the *Stra8* gene is a molecular marker of female germ cell sex differentiation. *Stra8* is expressed in ovarian germ cells and not testicular germ cells at E12.5, approximately one day before meiotic germ cells are first observed in the embryonic ovary (Fig. 3A). Furthermore, examination of *Stra8* expression in sex-reversed XY^{DOM} gonads has verified that *Stra8* positive cells are located in feminized regions of these gonads (Fig. 4). Thus, *Stra8* expression is correlated with the gonadal environment in which the germ cell develops rather than the sex chromosome content of the germ cell. This independence from sex chromosome constitution is in agreement with the expectations for a marker of germ cell sex differentiation. I believe that a prior report that *Stra8* is expressed in embryonic male germ cells at E12.5 and E14.5 may have resulted from improperly sexed tissue sections (Oulad-Abdelghani et al., 1996).

Stra8 is not the only gene that is known to be upregulated in female germ cells during gonadal development. Differential expression between embryonic male and female germ cells has been reported for two closely related X-linked genes, *Gpbox* and *Psx1* (Takasaki et al., 2000). These genes are expressed in male and female gonads from E10.5 to E15.5 with the highest levels evident at around E12.5. At E11.5 there is roughly two fold more *Gpbox* and *Psx1* in female gonads than in male gonads, while at E12.5 the difference has increased to approximately five fold. Unfortunately, the location of these genes on the X chromosome makes interpretation of the differential expression between male and female germ cells difficult. It is unclear whether this differential expression is a result of germ cell sex differentiation or whether it reflects X reactivation which occurs in XX germ cells shortly after the germ cells migrate into the gonad (Monk and McLaren,

1981; Tam et al., 1994). Reactivation of the inactive X chromosome in XX germ cells occurs with the same timing regardless of whether the XX germ cell is located in an ovarian or a testicular environment, and this process is thought to occur independently of germ cell sex differentiation (Jamieson et al., 1998; McLaren and Monk, 1981). Therefore, the two to five fold increase seen in XX germ cells could largely be explained by the location of *Gpbox* and *Psx1* on the X chromosome.

Additional research has revealed that *prostaglandin D2 synthase* (*Ptgds*) is upregulated in embryonic male germ cells by E13.5. Intriguingly, prostaglandin D2, the protstanoid generated by the PTGDS enzyme, exerts a masculinizing influence on the somatic cells of fetal ovaries grown in organ culture. Although *Ptgds* expression is initiated in the male genital ridge between E11.5 and E12.5, it is expressed in both the somatic Sertoli cells and in male germ cells and it is unclear at what stage male germ cells first express this gene.

Stra8 is upregulated in female germ cells in an anterior to posterior wave from E12.5 to E16.5 (Fig. 3). This wave encompasses the period within which female germ cells enter meiotic prophase, approximately E13.5 to E15.5. Since entry into meiosis is considered definitive evidence of female sex determination, I examined the developmental time course over which the meiosis specific gene *Dmc1* is expressed. Consistent with our *Stra8* analysis, I found that *Dmc1* positive germ cells are initially located primarily in anterior regions of the ovary (Fig 3). The anterior-to-posterior wave observed with *Stra8* appears to be more sharply defined than that observed with *Dmc1*. This may reflect the amount of time *Stra8* and *Dmc1* are expressed in any given cell. A

shorter window of expression for *Stra8* (as opposed to *Dmc1*) could produce an anterior-to-posterior wave that is visually more obvious.

Multiple lines of evidence have suggested that embryonic germ cells will enter meiosis cell autonomously unless the germ cell is exposed to a testicular environment. Therefore, my discovery that sex differentiation of female germ cells occurs as a wave in the embryonic ovary is unexpected. One might have predicted that germ cells would enter meiosis throughout the embryonic ovary in a random fashion. Given that differentiation of germ cells as female does not require an ovarian environment, what is the significance of the wave of sex differentiation that is observed? One possibility is that the germ cells that first reach the gonad during germ cell migration tend to be located in anterior regions, and the meiotic wave reflects either the time germ cells reach the gonad, or the number cell divisions that they have progressed through. However, studies that have examined the migration of primordial germ cells into the genital ridge have not documented the existence of such an anterior-to-posterior partitioning of arriving germ cells (Molyneaux et al., 2001), and I instead favor a second model in which meiotic entry reflects the state of ovarian somatic differentiation. I propose that as the ovarian soma differentiates, it becomes capable of promoting germ cell sex differentiation. In this model the promoting activity stimulates meiotic entry of germ cells, but is not required for this event to occur. The presence of meiosis stimulating activity in embryonic ovaries has previously been demonstrated (Byskov and Saxen, 1976). If somatic cells of the ovary are responsible for the meiotic wave, then differentiation of the ovarian soma should occur first in anterior portions of the ovary and later in posterior locations. Consistent with this supposition, I have recently observed that the upregulation of ovarian

somatic genes occurs in both central and anterior regions first and subsequently spreads to more posterior regions (*See Chapter 3*). A third model that I can not exclude is that a gradient of a meiosis activating substance is present in the ovary, and it is this gradient that generates the wave of female germ cell sex differentiation.

If sex differentiation of female germ cells and meiotic entry occur in an anterior to posterior wave, why hasn't this phenomenon been observed before? Most studies of embryonic ovaries have been carried out on sectioned tissue in order to perform the histology required to identify meiotic germ cells. This could be problematic for a number of reasons. High magnification is required to identify meiotic germ cells and this means that obtaining an overall impression of the location of these cells along the anterior-posterior axis may not be easy to achieve without a systematic approach. This problem is compounded by the fact that germ cells at multiple different stages of meiotic progression are present in the ovary and these stages can be difficult to strictly categorize (e.g., germ cells are often classified as "leptotene/zygotene" rather than "leptotene" or "zygotene"). These difficulties may be further complicated by the requirement for good sagittal sections at multiple developmental stages that would aid in the observation of an anterior-posterior wave. In addition, re-aggregation experiments that have been useful tools in the study of gonadal sex differentiation disrupt any anterior-posterior organization that might be present in the gonad. My visualization of specific subsets of differentiating germ cells by whole-mount *in situ* hybridization avoided these issues.

Stra8 mRNA is present in female germ cells at E12.5, just prior to meiotic entry, and is also abundantly expressed during the time period within which female germ cells first enter meiosis. Although I have not been able to determine whether *Stra8* expression

is maintained during the initial stages of meiosis, a prior analysis of *Stra8* expression in normal adult mouse tissues demonstrated that *Stra8* is specifically expressed in a subset of spermatogonia and possibly preleptotene spermatocytes (Oulad-Abdelghani et al., 1996). The STRA8 protein lacks obvious homologies to other known protein sequences. Nonetheless, it seems likely that the expression of *Stra8* is an indicator of a male or female germ cell's readiness to progress into meiotic prophase. A recent report establishing that female germ cell sex determination occurs by E13.5 raises the possibility that upregulation of *Stra8* may be closely linked to the events that underlie this process. Functional analysis will reveal whether *Stra8* plays a role in either of these events.

Materials and Methods

Mice and Embryo Collection

Mouse embryos derived from matings between male and female C57BL/6 mice purchased from Taconic Farms Inc. (Germantown, NY) were used in all experiments except those using sex-reversed XY^{DOM} gonads. For the generation of sex-reversed XY^{DOM} gonads, male Zalende/Ei mice from Jackson Lab (Bar Harbor, ME) were bred with C57BL/6 females to generated F1 hybrids. F1 stud males were mated to C57BL/6 females to create XY^{DOM} embryos with partially or fully sex-reversed gonads as previously reported (Eicher and Washburn, 1983). Timed matings were performed with noon on the day a vaginal plug was found designated as E0.5. Germ cell depleted embryonic gonads were generated by injecting pregnant females at E9.5 with 0.2 ml of 6.6 mg/ml busulfan in 50% DMSO (~0.053 mg busulfan per gram body weight)(Merchant, 1975).

Embryos for *in situ* hybridization were collected at E11.5-E16.5 and the genital ridges were dissected out in PBS and subsequently fixed in 4% paraformaldehyde at 4°C overnight. Tissues were dehydrated into 100% methanol and stored at -20°C until used. Embryos younger than E12.5 were sexed by isolating DNA and using a Y chromosome specific PCR assay (primer sequences: T35L-5'GGAATGAATGTGTTCCATGTCG –3'; T35R-5'-CTCATGTAGACCAAGATGACC-3'). Gonads isolated from E12.5-E16.5 were sexed visually with the exception of E14.5 XY^{DOM} gonads derived from Zalende/Ei/C57BL/6 F1xC57BL/6 matings which were sexed using the Y chromosome PCR assay.

RT-PCR

Total RNA was isolated from male and female genital ridges using Trizol (GIBCO-BRL) as directed by the manufacturer. 1 μg of total RNA was reverse transcribed with oligo $d(T)_{18}N$ using Superscript II (GIBCO-BRL) in a total reaction volume of 25 μl. PCR was performed using 1 μl of RT as template in a total volume of 20 μl (10 mM Tris-HCl (pH 9), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 0.2 mM dNTPS, 0.5 μM primers, 0.5 U Taq polymerase). PCR cycling conditions for all primers were as follows: 94°C (30 seconds), 60°C (30 seconds), 72°C (1 minute) for 25-30 cycles. *Stra8* primers: 6695 (5'- GAGGTCAAGGAAGAATATGC-3') and 6698 (5'-CAGAGACAATAGGAAGTGTC-3'), *Gapd* primers: 6787 (5'-GTGTTCCTACCCCCAATGTG-3') and 6788 (5'-GTCATTGAGAGCAATGCCAG-3'), *Mis* primers: 4616 (5'-TTGCTGAAGTTCCAAGAGCC-3') and 4617 (5'-TTCTCTGCTTGGTTGAAGGG-3').

***In situ* hybridization**

Digoxigenin whole-mount *in situ* hybridizations were performed essentially as previously reported (Wilkinson and Nieto, 1993). Digoxigenin riboprobes were generated for *Stra8* and *Dmc1* as follows: A 514 bp fragment of *Stra8* (corresponding to nucleotides 766-1279 of the Stra8 cDNA; GenBank Accession Number: NM009292) was amplified by RT-PCR and inserted into the TA cloning vector pCR2.1-TOPO (Invitrogen). *Dmc1* riboprobes were made by amplifying a 644 bp fragment of Dmc1 cDNA (602-1245 of NM010059) and cloning it into TA cloning vector pCR4-TOPO (Invitrogen). Plasmids were linearized and transcribed with T3 or T7 RNA polymerase in the presence of Dig-

labeling mix (Roche) to create digoxigenin riboprobes. Fluorescein *Stra8* riboprobe was created using Fluor-labeling mix (Roche).

Double *in situ* hybridization was performed by hybridizing embryonic gonads simultaneously with fluorescein labeled *Stra8* riboprobe and digoxigenin labeled *Tet4* riboprobe. Digoxigenin riboprobe signals were detected by incubation with anti-Dig-AP followed by BM Purple (Roche). Anti-Dig-AP was inactivated with 0.1 M Glycine-HCl (pH 2.2) and fluorescein riboprobe signals were detected with anti-Fluor-AP (Roche) and INT/BCIP (Pharmingen).

Alkaline Phosphatase Staining of Germ Cells

Embryonic gonads were dissected out in PBS and placed in 70% ethanol at 4°C overnight. Gonads were rinsed three times in NTMT (100 mM Tris-HCl pH9.5, 100 mM NaCl, 50 mM MgCl, 0.1% Tween-20) after which endogenous AP activity was detected by incubation at room temperature in NTMT containing 0.337 mg/ml NBT and 0.175 mg/ml BCIP (Roche).

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Chapter 5

Stra8 is Required for Meiotic Progression in Males and Females

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Abstract

To a great extent the study of mammalian meiotic prophase has been limited to elements that are evolutionarily conserved from yeast to man. While this work has proved quite valuable, we remain ignorant of most mammalian specific components of this developmental process. The *Stra8* gene encodes a protein of unknown function that is expressed at premeiotic or early meiotic stages of oogenesis and spermatogenesis in mice. We have been unable to identify genes homologous to *Stra8* in any non-vertebrate species nor in the sequenced genome of the fish *Fugu rubripes*. Thus, *Stra8* may be specific to mammals and perhaps to certain other vertebrate lineages. Moreover, a search for proteins similar to STRA8 in the nearly complete mouse and human genomes has failed to reveal related proteins. This suggests that STRA8 may play a unique and non-redundant role in mammalian germ cell development. In order to investigate the requirement for STRA8 in mammalian germ cells, we have disrupted the *Stra8* gene in mice. Our preliminary analysis has revealed that *Stra8* null males and females display abnormal germ cell development. At embryonic day 15.5 (E15.5), female germ cells of -/- animals do not express the meiotic prophase gene *Dmc1* which is abundantly expressed in +/- and +/+ oocytes at this stage. In addition, at 14 days postpartum (14 dpp) -/- ovaries are greatly reduced in size and contain only a small number of follicles. The testes of -/- males are also significantly reduced in size by 24 dpp. Other than germ cell defects, *Stra8* null mice appear to be fully viable and phenotypically unremarkable. Further analysis of the germ cell defects observed in these mice is ongoing.

Introduction

We have recently observed that embryonic ovarian germ cells differentiate and enter meiosis in an anterior-to-posterior wave. This striking differentiation pattern was unexpected, given prior evidence suggesting that meiotic entry of germ cells occurs cell autonomously in the absence of a testicular environment. This wave of differentiation was initially defined by the expression pattern of *Stra8*, a germ cell gene that was previously reported to be expressed in spermatogonial stem cells of the postnatal mouse testis (Oulad-Abdelghani et al., 1996). We subsequently demonstrated that the meiotic gene *Dmc1* is upregulated and expressed in a similar anterior-posterior wave (*see Chapter 4*).

Our observation that *Stra8* is expressed in ovarian germ cells during the time period that female germ cells enter meiosis suggests a possible role for Stra8 in early meiotic or premeiotic stages of oogenesis. Moreover, the expression of *Stra8* in a subset of spermatogonial cells of the adult testis implies that Stra8 may play a similar role in spermatogenesis. It has also been suggested that Stra8 may have a placental function as well, since *Stra8* expression has been observed the giant cells of the trophoblast (Sapin et al., 2000).

Stra8 was originally isolated in a screen to identify genes that are upregulated in the P19 embryonic carcinomal cell line upon exposure to retinoic acid (Bouillet et al., 1995). The mouse *Stra8* gene encodes a 393 amino acid protein. The most obvious feature of this protein is a centrally located domain that is rich in glutamic acid. Alignment of the mouse protein with the electronically predicted human and rat orthologs reveals that this domain is quite variable (Figure 1). At

Figure 1. Alignment of mouse, rat, and human STRA8 protein sequences. The mouse sequence is derived from the published cDNA sequence while the rat and human sequences were electronically predicted from genomic sequence.

Species	Start	Sequence	End
Mouse	1	MATPGEGNQPSDDGAPQPLAQLQKLEPRVVRRRLSQ	36
Rat	1	MATPGEGNQPRDDGAPQPVPQLQQLEPRVARRRLSQ	36
Human	1	MATPEENSNPHDRATPQLPAQLQELEHRVARRRLSQ	36
Mouse	37	ARHRATLVGLFNNLRKAVYSQSDITASKWQVLNRTK	72
Rat	37	ARHRATLVGLFNNLRKTVYSQSDLTASKWQVLNKAK	72
Human	37	ARHRATLAALFNNLRKTVYSQSDLIASKWQVLNKAK	72
Mouse	73	IHIQEQEESLDKLLKLKASFNLQDGNPNSLEEVKEE	108
Rat	73	SHIQELEGSLDNLLKLKGFFNLQDGNPNTLEEVKEE	108
Human	73	SHIPELEQTLDNLLKLKASFNLEDGHASSLEEVKKE	108
Mouse	109	YARMYSENDSVFLNSFLQDSPPEWFPSEAVGPDAEE	144
Rat	109	YARMYSENESVFLNSFPQNSPPAWLPPEAVGPDAEE	144
Human	109	YASMYSGNDSLLSNSFPQNGSSPWCPTEAVRKDAEE	144
Mouse	145	EGEEEGEEEGEEGEEEEEGDEEGEEEEENGEEREVE	180
Rat	145	GG----------------------------NDEEREVE	154
Human	145	EEDEEEEDQ---------------EEEEEEEEEEEE	165
Mouse	181	EYQEEEEEEEEEEKKVDLSHSSSTLLPDLMEFERYL	216
Rat	155	EYQEEEEEEEEE-KKVDLSHSSPTLLPDLMEFERYL	189
Human	166	EEEEEEEEEEEEEKKVILY-SPGTLSPDLMEFERYL	200
Mouse	217	NFYKQTMDLLTMNSIISAHEVTLPIVSAAISHLWQT	252
Rat	190	NFYKQTMDLLTMNSIISAQEVTLPIVSAAISHLWQT	225
Human	201	NFYKQTMDLLTGSGIITPQEAALPIVSAAISHLWQN	236
Mouse	253	LSEEKKARLLQVWEQQHSAFADLTEACLELAGVEGS	288
Rat	226	LSEEKKARLLQVWEQQHSTFSDLTEACLELAGAEGS	261
Human	237	LSEERKASLRQAWAQKHRGPATLAEACREPACAEGS	272
Mouse	289	MKDSGVDSQGASCSLESTPEEILFEDAFDVASFLDK	324
Rat	262	MKDSGVDSQGASCSLDSTPEEILFEDAFDVASFLDK	297
Human	273	VKDSGVDSQGASCSLVSTPEEILFEDAFDVASFLDK	308
Mouse	325	SEAQHMSNISAMFATCNSENPEEKFQLYIQIIEFFK	360
Rat	298	SEAQRLSNISAVFASCNSENLEEKFQLYIQIIEFFK	333
Human	309	SEVPSTSSSSSVLASCNPENPEEKFQLYMQIINFFK	344
Mouse	361	SLGCVNTPLNQEPEPPDDDDAMLLKCLETFDDL--	393
Rat	334	GLGCVNTPLNQEPDPPVDDDVMLLKCLETFDDEDL	368
Human	345	GLSCANTQVKQEASFPVDEEMIMLQCTETFDDEDL	379

the amino acid level mouse STRA8 is 89% identical to its rat ortholog and 71% identical to its human ortholog. Therefore, Stra8 shows significant divergence between these mammalian species. STRA8 does not exhibit convincing homology to any known proteins and searches against the human and mouse genomes has failed to identify other related genes. Similarly, we have been unable to identify homologs in *Drosophila*, *C. elegans*, *S. cerevisiae*, and *Fugu rubripes*. Thus, *Stra8* has either diverged to such an extent that we can not identify homologs in these species, or perhaps *Stra8* is unique to certain vertebrate lineages.

The apparent lack of related genes suggests that Stra8 may play a unique role in mammalian germ cell development. In order to determine the functional requirement for Stra8 during male and female germ cell development we have disrupted the mouse *Stra8* gene.

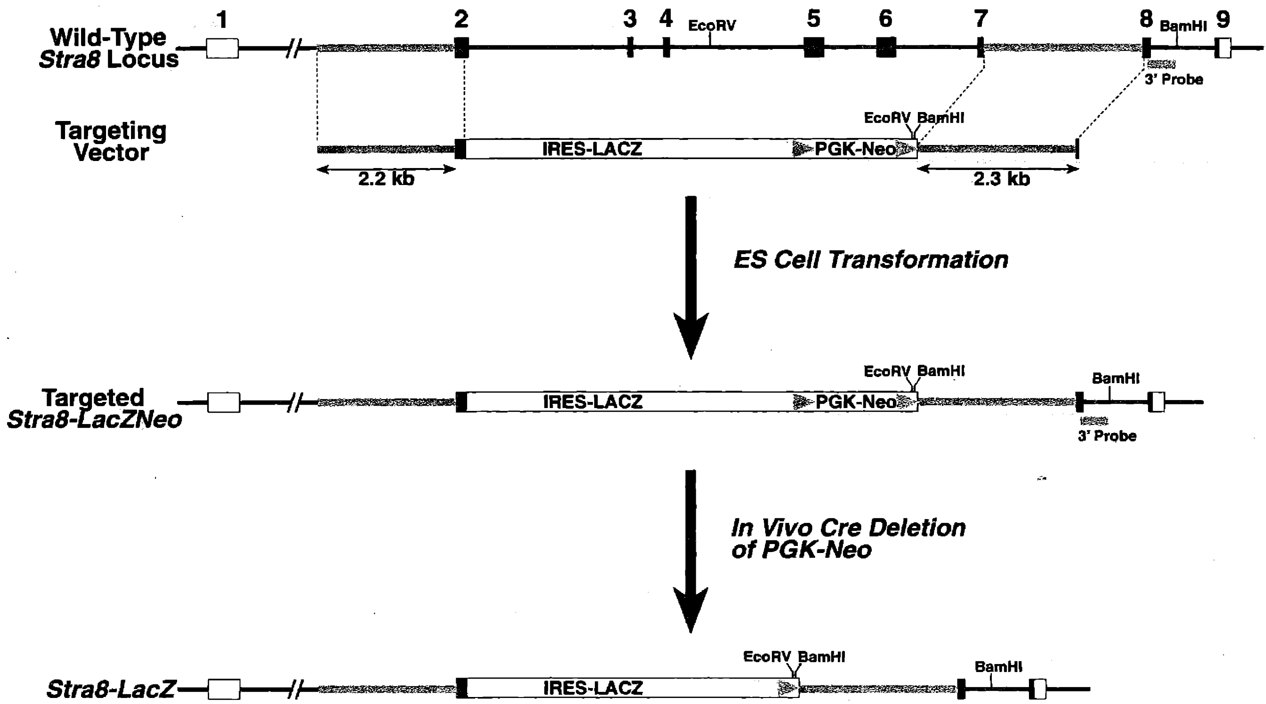
Results

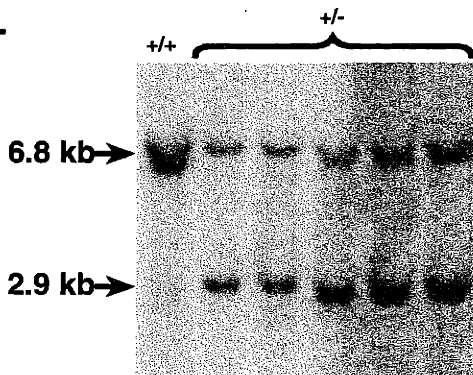
Targeted disruption of *Stra8*

Stra8 is a reasonably compact nine exon gene with coding sequence contained within exons 2 to 9 (Fig. 2A). We disrupted the *Stra8* gene by inserting a 6.7 kb *IRES-LacZ/PGK-Neo* selection cassette and deleting a 7.8 kb region that contained the majority of *Stra8* coding sequence including part of exon 2 and all of exons 3 through 7. The *IRES-LacZ* contains an internal ribosomal entry site that allows translation of the *LacZ* open reading frame even when upstream initiation codons are present (Jang et al., 1988; Takeuchi et al., 1995). We fused the *IRES-LacZ* with exon 2 of *Stra8* in the hope that *LacZ* would be incorporated into the mature *Stra8* transcript and be expressed using the endogenous *Stra8* promoter. We isolated multiple homologously targeted ES cell clones (Fig. 2B), and successfully achieved germline transmission from three independent cell lines (Fig. 2C).

We are currently in the initial stages of characterizing the phenotype of *Stra8* null mice. *Stra8* +/- heterozygous males and females are fully viable and fertile. Our initial observations of *Stra8* -/- mice generated by +/- crosses indicate that -/- mice are also viable and occur at the expected Mendelian ratio. Therefore, the placental expression of *Stra8* does not appear to be necessary to support embryonic development. However, examination of -/- ovaries from 14 day postpartum (14 dpp) females has revealed gross morphological abnormalities. -/- ovaries are significantly reduced in size as compared to +/- littermates (Fig. 3A). Only a small number of large primary follicles are apparent in -/- ovaries and no primordial follicles are evident (Fig. 4B, D, and F). In contrast, +/- ovaries have many developing follicles of various sizes (Fig. 4A, C, and E).

Figure 2. Genomic structure of *Stra8* and knockout strategy. A) The Stra8 locus was disrupted by deleting a 7.8 kb fragment and inserting a *IRES-LacZ/PGK-Neo* selection cassette. B) Homologously targeted ES cell clones were confirmed by Southern blot analysis using a BamHI/EcoRV double digest and an external 3' probe. The wild-type allele produces a 6.8 kb band, while the homologously targeted allele produces a 2.9 kb band. C) A multiplex PCR assay was used to confirm germline transmission of the targeted *Stra8* allele.


A.
Wild-Type Stra8 Locus
1
2
3
4
EcoRV
5
6
7
8
BamHI
9
3' Probe
Targeting Vector
EcoRV BamHI
IRES-LACZ
PGK-Neo
2.2 kb
2.3 kb
ES Cell Transformation
Targeted Stra8-LacZNeo
EcoRV BamHI
IRES-LACZ
PGK-Neo
BamHI
3' Probe
In Vivo Cre Deletion of PGK-Neo
Stra8-LacZ
EcoRV BamHI
IRES-LACZ
BamHI


B.
+/+
+/-
6.8 kb
2.9 kb

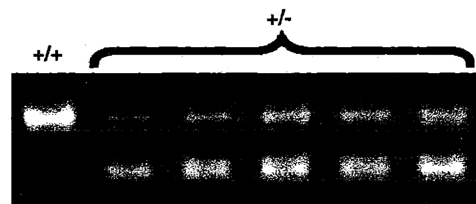

C.
+/+
+/-

Figure 3. Gross abnormalities and gene expression defects in *Stra8* null gonads. A) At 14 dpp -/- ovaries are greatly reduced in size and in number of follicles relative +/- ovaries. B) No gross morphological differences are observed between +/- and -/- ovaries at E15.5. However, whole-mount *in situ* hybridization reveals a lack of *Dmc1* expression in -/- ovaries. C) No size difference is observed between +/- and -/- testes at 14 dpp. D) By 24 dpp -/- testes are significantly reduced in size relative to +/- littermates. Note that all four panels are taken at different magnifications.

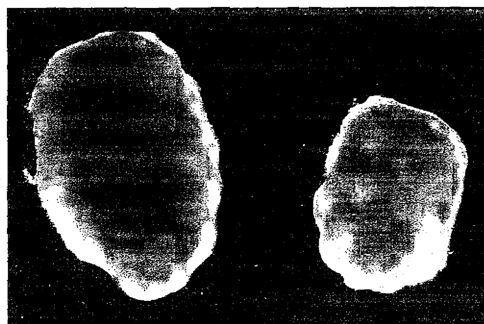
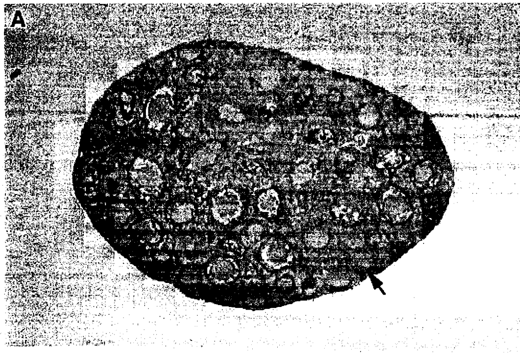


Figure 4. H&E stained sections from *Stra8* +/- and -/- 14 dpp ovaries. Many primary follicles are observed in +/- ovaries (A), while few follicles are present in -/- ovaries (B). At higher magnification primordial follicles are seen in +/- (C), but not -/- (D) ovaries. Magnified view of primary follicles in +/- (E) and -/- (F) ovaries. Arrows in (A) and (B) indicate primary follicles. Arrows in panel (C) indicate primordial follicles.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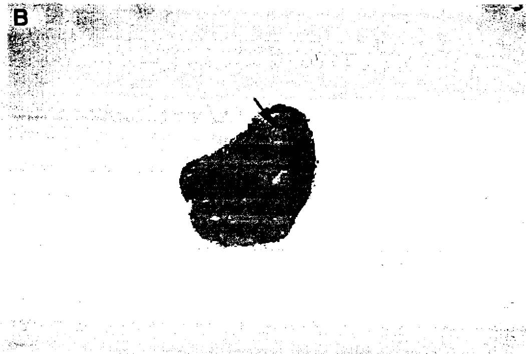
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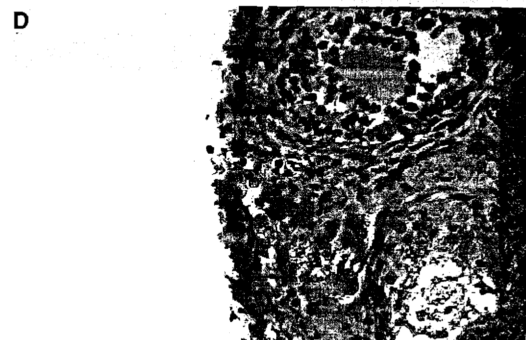
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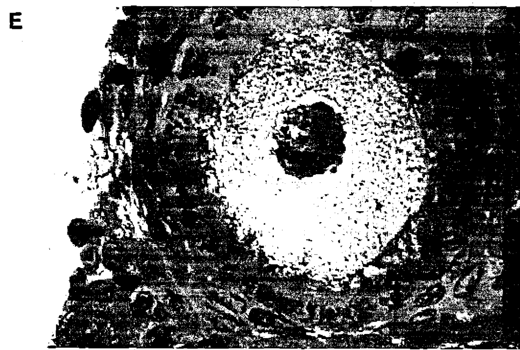
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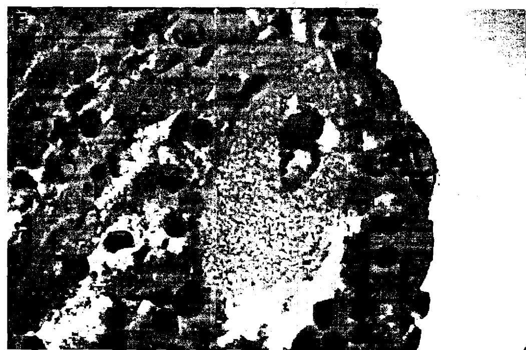
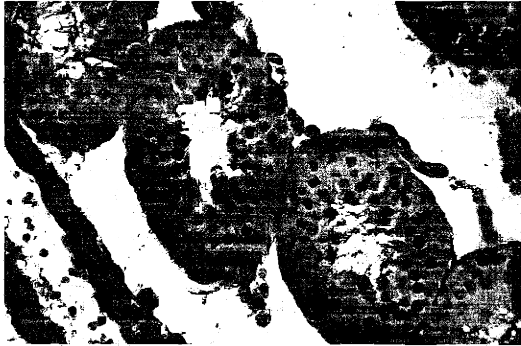
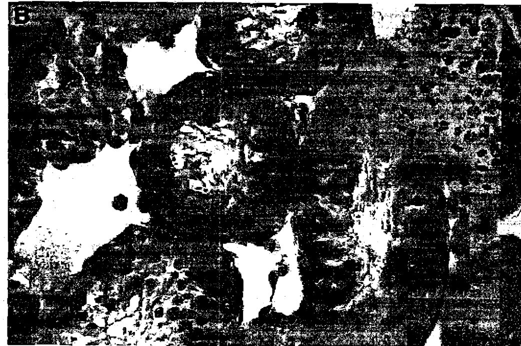


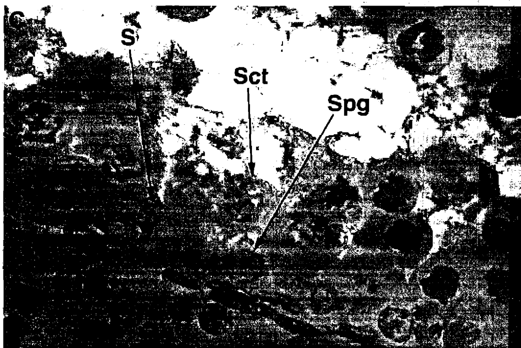
We have also examined -/- ovaries at embryonic day 15.5 (E15.5). At this stage *Stra8* -/- ovaries are outwardly indistinguishable from heterozygous and wild-type littermates. To determine whether meiotic germ cells are present in E15.5 -/- ovaries, we examined the expression of *Dmc1* by whole-mount *in situ* hybridization. *Dmc1* is expressed during meiotic prophase and is normally abundantly expressed in female germ cells at E15.5. In contrast to +/+ and +/- ovaries, we observed no expression in -/- E15.5 ovaries (Fig. 3B). This suggests that there are few or no meiotic germ cells present at this stage. However, we do not yet know what differentiation state the germ cells are in, or even whether the germ cells are dead or dying. It is worth noting that since we do observe some follicles in 14 dpp ovaries, it seems likely that at least a few germ cells do at least partially progress through meiosis.

The testes of -/- males do not exhibit abnormalities that are externally obvious at 14 dpp and appear identical to the testes of +/- littermates (Fig. 3C). However, sections of 14dpp -/- testis reveal greatly reduced numbers of germ cells and very few primary spermatocytes relative to heterozygous animals (Fig. 5). By 24 dpp the testes of -/- animals are significantly smaller than those of +/- littermates (Fig 3D). In males, meiosis is normally initiated at ~10 dpp. Between 10 dpp and five weeks of age mouse testes undergo a large increase in size as the seminiferous tubules fill with meiotic and post-meiotic germ cells. Therefore, the reduced testis size observed in 24 dpp Stra8 -/- males is undoubtedly due to a reduced number of meiotic germ cells.

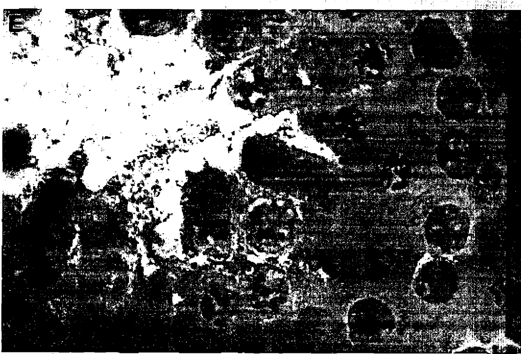
Figure 5. H&E stained sections for 14 dpp *Stra8* +/- and -/- testes. Many spermatogonia (Spg), meiotic spermatocytes (Sct), and Sertoli cells (S) are present in *Stra8* +/- testes ((A), (C), and (E)). *Stra8* -/- testes contain many Sertoli cells, but very few spermatogonia and primary spermatocytes ((B), (D), and (F)).

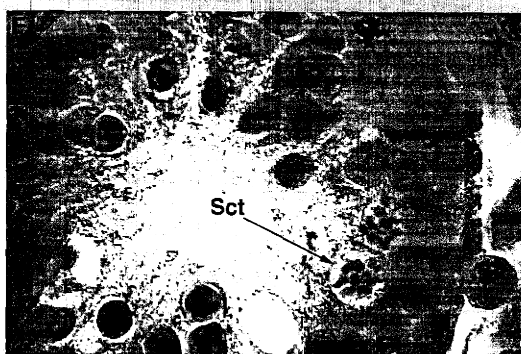





S
Sct
Spg

D
Spg
S




Sct

Future analysis

We plan to continue our characterization of the *Stra8* knockout mice using several techniques. We have placed the highest priority on obtaining histological sections from male and female gonads throughout mouse development including embryonic and postnatal periods. This will help us to determine at what stage defects are first apparent and when meiotic germ cells are actually present. Since *Stra8* is upregulated in female germ cells from E12.5-E16.5 we will initially focus on this developmental time period in females. *Stra8* is not expressed in male germ cells until approximately the time of birth, so we will concentrate on postnatal stages in our examination of *Stra8* -/- male germ cells.

We also plan to examine the expression of *Dmc1* (Pittman et al., 1998; Yoshida et al., 1998), *Spo11* (Baudat et al., 2000; Romanienko and Camerini-Otero, 2000) and other genes that are expressed upon meiotic entry to determine whether *Stra8* -/- female germ cells initiate expression of these genes at any developmental stage. The failure of *Stra8* null germ cells to express these genes would indicate an extremely early meiotic or premeiotic defect and could represent the earliest meiotic block that has been produced in mammals. We will also look for the expression of *prostaglandin D2 synthase* (Adams and McLaren, 2002) and other markers of male germ cell identity (M. Wu and D. Page, unpublished results) to determine if there are defects in female germ cell sex differentiation. In addition, we will perform TUNEL assays determine whether germ cells are undergoing apoptosis. Since *Stra8* is expressed in an anterior-to-posterior wave in the embryonic ovary, it is possible that we will observe a similar wave of cell death. In other meiotic mutants, such as *Dmc1* (Romanienko and Camerini-Otero, 2000) and *Spo11*

(Baudat et al., 2000; Romanienko and Camerini-Otero, 2000), it has been observed that the majority of germ cell death can occur days after the time at which the expression of these genes is normally required. We will have to determine whether a similar result is observed in *Stra8* null mice. As the precise nature of the *Stra8* null phenotype comes into better focus, we will obviously adjust our experimental approach accordingly. Though the characterization of our *Stra8* knockout mice has just begun, it seems apparent that this mutant will yield intriguing insights into mammalian germ cell biology.

Materials and Methods

Generation of targeted ES cells

We constructed a *Stra8 IRES-LacZ/PGK-Neo* targeting construct using PCR products amplified with Advantage HF2 polymerase (Clontech). All PCR products were sequenced to ensure the absence of point mutations. The v6.5 ES cell line (Rideout et al., 2000) was electroporated with 40 μg of linearized *Stra8 IRES-LacZ/PGK-Neo* targeting construct and selection was performed with 300 μg/ml G418 (GIBCO-BRL). ES cell colonies were picked and screened by using a long distance PCR assay that was specific to clones that are correctly targeted at the 5' arm. Southern blot analysis was used to confirm homologous targeting at the 3' arm. ES cell clones were injected into Balb/c or C57Bl/6 blastocyts and transferred to pseudopregnant Swiss Webster females. Germline transmission was obtained from three independent clones.

Acknowledgements

We thank Rudolf Jaenisch for the V6.5 ES cell line and Alex Bortvin and members of the Jaenisch lab for ES cell targeting reagents and advice.

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Rideout, W., Wakayama, T., Wutz, A., Eggan, K., Jackson-Grusby, L., Dausman, J., Yanagimachi, R., and Jaenisch, R. (2000). Generation of mice from wild-type and targeted ES cells by nuclear cloning. Nat Genet *24*, p109-10.

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Chapter 6

Characterization and Targeted Disruption of *Tet14*: A Small X-linked Gene Expressed in Testis

Douglas B. Menke, Mary L. Goodheart and David C. Page

Abstract

We previously reported the isolation of multiple genes that are expressed in a sexually dimorphic pattern during the differentiation of embryonic testes and ovaries. Here we describe one of these genes in greater detail and report our progress on the functional characterization of this gene. *Tet14* (day **T**welve and half **E**mbryonic **T**estis, transcript 14) is a small novel gene that is specifically expressed in embryonic and adult mouse testes. The *Tet14* transcript is detectable in the developing testicular cords by embryonic day 12.5 (E12.5) and encodes a putative open reading frame of 59 amino acids. Though this open reading frame lacks homology to previously characterized proteins, we have identified a related mouse gene *Tet14-like*. Both *Tet14* and *Tet14-like* map near to one another on the mouse X chromosome, and their human orthologs map to human Xq26. We have generated a conditional knockout of *Tet14* to test the functional requirement for this gene during mouse development. We have recently achieved germline transmission and are currently breeding these mice to generate male and female knockout mice for phenotypic analysis.

Introduction

Historically, the functional analysis of genes required for testis development has been driven largely through the identification of loci responsible for human syndromes that result in partial or complete sex-reversal (Bardoni et al., 1994; Foster et al., 1994; Pelletier et al., 1991; Sinclair et al., 1990; Wagner et al., 1994). More recently, insights into ovarian and testicular differentiation have been gained through the unexpected observation of gonadal defects in mouse knockouts of the *Wnt4* and *Fgf9* genes (Colvin et al., 2001; Vainio et al., 1999). We have taken an alternative approach to identify candidate genes that may be involved in testicular and ovarian differentiation by isolating genes that are differentially expressed between embryonic testes and ovaries (*see Chapter 2*).

Using a PCR-based cDNA subtraction we previously identified multiple genes that are upregulated in embryonic testis relative to embryonic ovary at embryonic day 12.5 (E12.5). We selected one of these genes, *Tet14*, to further characterize. *Tet14* is the most testis specific of the differentially expressed genes we identified and encodes a small novel transcript. Our initial observations of *Tet14* expression by RT-PCR indicated that *Tet14* is expressed in embryonic testis from E12.5 to E14.5 and is abundantly expressed in testis at 8 days postpartum and in germ cell depleted adult testis. In addition, whole-mount *in situ* hybridization revealed that at E13.5 *Tet14* is expressed within the testicular cords. Given the intense RT-PCR signal observed in germ cell depleted adult testis the most likely source of *Tet14* expression is Sertoli cells, the somatic cells of the testicular cords (*see Chapter 2*).

Results

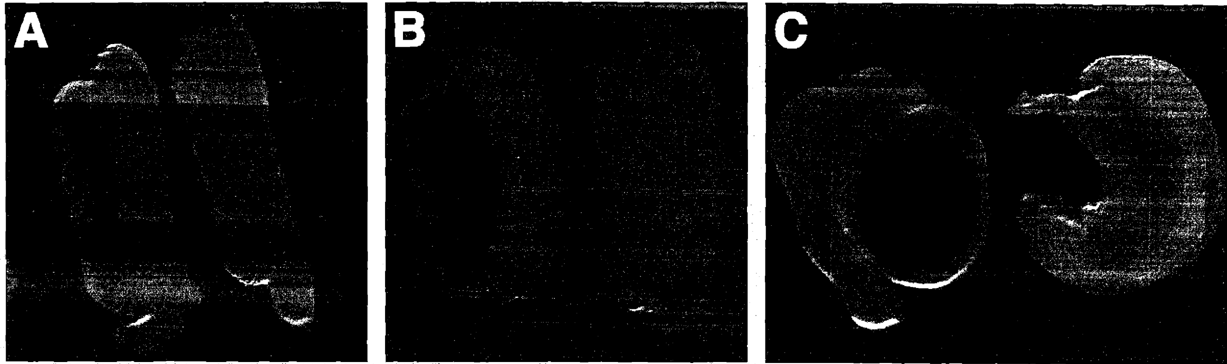
Tet14 is upregulated in embryonic testes by E12.5

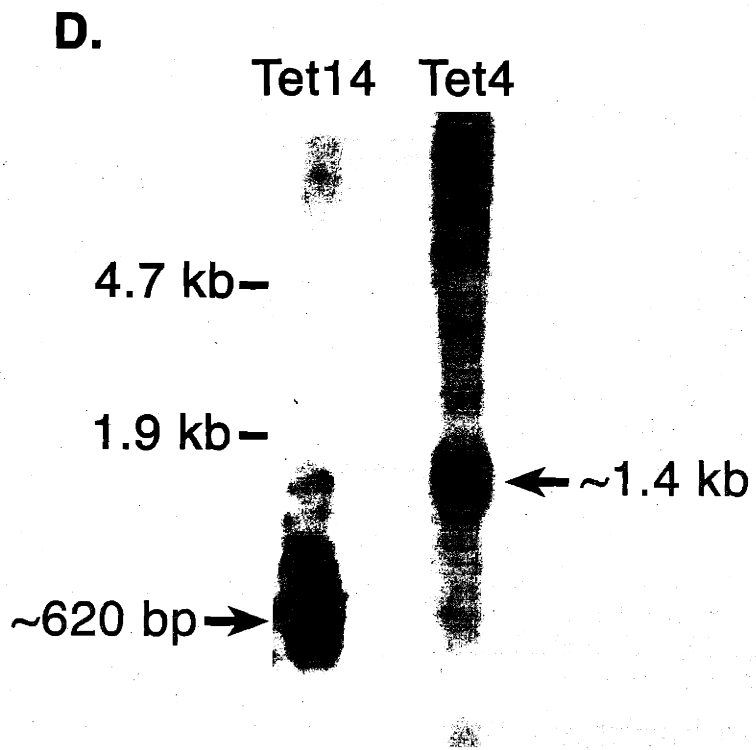
We performed additional whole-mount *in situ* hybridizations on XX and XY gonads to better characterize the expression pattern of *Tet14*. In agreement with our prior observations, we find that expression of *Tet14* is confined to the testicular cords of the testis at E12.5, E13.5, and E15.5 (Fig. 1A, B, and C). Sectioning of these whole-mounts revealed staining within the testicular cords, however the signal was too diffuse to determine whether the signal originated from Sertoli cells and/or germ cells (data not shown). However, *Tet14* was not detected in E12.5 FACS purified germ cells by RT-PCR (data not shown). We were unable to detect *Tet14* in XX or XY gonads at E11.5 by *in situ* hybridization (data not shown). However, we did detect *Tet14* expression is in XY gonads at E12.0 by RT-PCR (data not shown).

Tet14 encodes a small novel open reading frame

When we originally identified *Tet14* in our embryonic testis cDNA subtraction, we screened a conventional cDNA library derived from E12.5 testis and ovary to isolate multiple *Tet14* cDNA clones. The consensus cDNA sequence generated by these cDNA clones was 478 bp in length and contained a putative open reading frame (ORF) of 59 amino acids. Our attempts to extend this sequence via 5' RACE failed to produce additional 5' sequence. Due to the small size of this transcript and its lack of homology to known proteins, we determined the size of the *Tet14* mRNA by performing Northern blot analysis on adult testis total RNA (Fig. 1D). Hybridization with a *Tet14* probe revealed an abundant small transcript approximately 600-650 bps in length. This size range is

Figure 1. Expression of *Tet14* in embryonic gonads and adult testes. Whole-mount *in situ* hybridization of *Tet14* on embryonic testes and ovaries at A) E12.5, B) E13.5, and C) E15.5. D) Northern blot analysis on adult testis total RNA probed with *Tet14* reveals a 600-650 bp band. The blot was stripped and re-probed with a *Tet4* probe to confirm the size estimated for the *Tet14* band. The full-length *Tet14* cDNA is 478 bp and the full-length *Tet4* cDNA is ~1300 bp.


A
B
C


D.
Tet14
Tet4
4.7 kb
1.9 kb
~1.4 kb
~620 bp

appropriate for polyadenylated *Tet14* transcripts, and we conclude that we have, in fact, isolated full-length *Tet14* cDNA.

Though the 59 a.a. ORF contained within the *Tet14* transcript exhibits no homology to characterized proteins, we did identify one related mouse transcript and two related human transcripts in the EST databases. One of the human transcripts is represented by multiple human testis ESTs and contains an ORF of 59 a.a. that is 50% identical to mouse TET14 (Fig. 2A). We believe that this human gene is the ortholog of mouse *Tet14*. The related mouse transcript, which we have named *Tet14-like*, is represented by ESTs from 2-cell embryos, E10.5 embryos, placenta and testis and encodes an ORF of 94 a.a. that is 41% identical to Tet14 over its first 60 a.a (Fig. 2B). The second human gene contains a 99 a.a. ORF that is 36% identical to mouse TET14-like and is represented by ESTs derived from kidney, melanocyte, brain, prostate, testis, and germ cell tumors. Despite the low identity, we believe that this human transcript is orthologous to *Tet14-like* (*see below*).

***Tet14* and *Tet14-like* are X-linked**

We have mapped the human *TET14* and *TET14-Like* genes to the distal long arm of the human X chromosome (Xq26, data not shown). Sequence produced by the human genome project has revealed that these genes are positioned immediately adjacent to each other in a head-to-head arrangement (Fig. 3). Surprisingly, the 5' most cDNA sequences for these genes are separated by a mere 479 bps. Both *TET14* and *TET14-like* consist of two exons with their putative ORFs contained entirely within the second exon. Small local duplications have produced at least three additional copies of *TET14* and *TET14-*

Figure 2. Amino acid alignments of the 59 amino acid open reading frame of mouse TET14 with related human and mouse sequences. A) Alignment of mouse and human *Tet14* ORFs. B) Alignment of mouse and human TET14 and TET14-like ORFs.

A.

```
mTet14        1   M D E K N P E A V P R P P E Q N T E L V P P K K S K S K K P   30
hTET14        1   M D K E V P K G S P R E P A L N I K K S D - K S F K R K K P   29

mTet14       31   - A N I L I Y L I D R H L G R P R N D M D L F E W V W T L K   59
hTET14       30   T E N V L I F L I N R Q L G R H R S D I D L S R W V W M L S   59
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B.

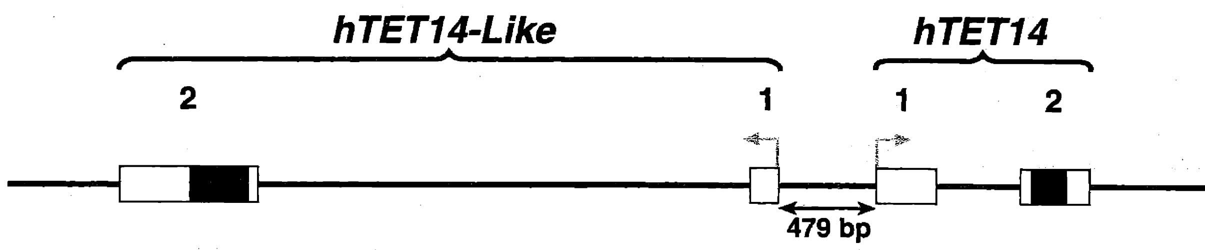
```
mTet14        1   M D E K N P E A V P R P P E Q N T E L V P P K K S K S K K P   30
hTET14        1   M D K E V P K G S P R E P A L N I - K K S D K S F K R K K P   29
mTet14-Like   1   M D E R Q H E A V P R P A R Q S T S R K A D E Y L K P S L G   30
hTET14-Like   1   M A Q G H S E V G P S T S A W S I R R K V D E Y L R P S L A   30

mTet14       31   - A N I L I Y L I D R H L G R P R N D M D L F E W V W T L K   59
hTET14       30   T E N V L I F L I N R Q L G R H R S D I D L S R W V W M L S   59
mTet14-Like  31   K K N L M S F L L G R Q L G R Q K S D S E M S K W L W M V T   60
hTET14-Like  31   S K S V L S V L L S R Q L G N H R N D V D L T E W L W T L K   60

mTet14        0   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -   59
hTET14        0   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -   59
mTet14-Like  61   R G T P G W S W P L P N H H I T Y M L M P T P N H S K C F Q   90
hTET14-Like  61   Q A I P G W T W P L P T R H T I N P P L P P G E D G K S K K   90

mTet14        0   - - - - - - - - - -                                            59
hTET14        0   - - - - - - - - - -                                            59
mTet14-Like  91   K S G K - - - - - -                                            94
hTET14-Like  91   M V W I N K S F P                                              99
```

Figure 3. Genomic arrangement of the human *TET14* and *TET14-Like* genes. Green arrows indicate the 5' most region of exon 1 for *TET14* and *TET14-Like* and the direction of transcription. The open reading frames of *TET14* and *TET14-Like* are located in the second of exon of each gene and are indicated in black.



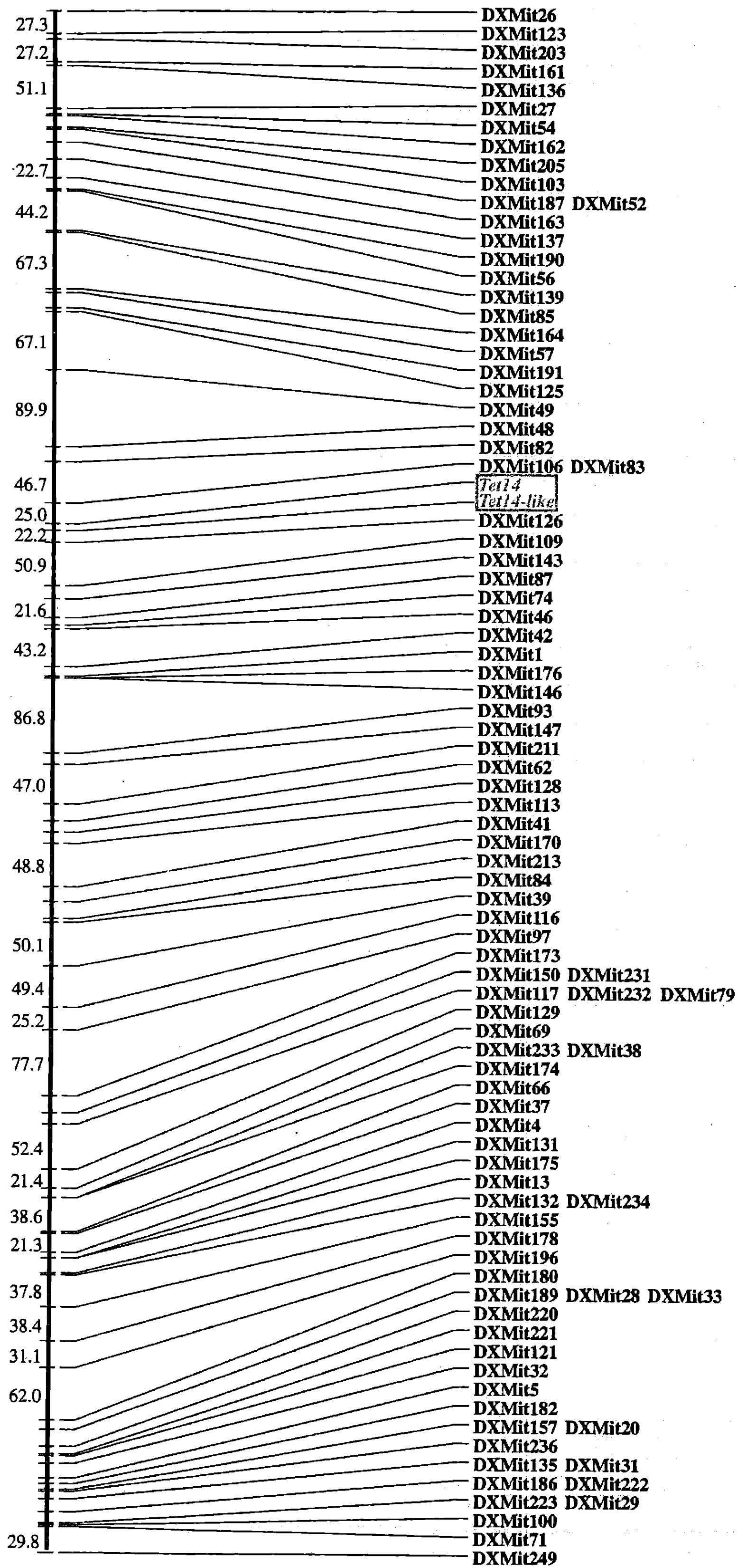
hTET14-Like
hTET14
2
1
1
2
479 bp

Like at Xq26 that exhibit ~70-89% nucleotide identity to each other. It is not clear whether any of these additional copies are functional genes. However, only one of these *TET14* copies is represented by ESTs and this copy has a disrupted open reading frame. Other than these duplications of the *TET14/14-Like* locus, we have found no other sequences in the human genome that contain clear homology to mouse or human *Tet14* or *Tet14-Like*.

The mouse genes, *Tet14* and *Tet14-like*, also map near to each other on the proximal region of the mouse X chromosome (Fig. 4). We sequenced the *Tet14* genomic locus and found that mouse *Tet14* is a two exon gene that contains an 8.1 kb intron (Fig. 5A). The putative ORF is contained entirely within the second exon, as we observed for the orthologous human gene. Using a combination of sequence that we generated in house and shotgun sequence from the mouse genome project we have examined 17.6 kb of sequence that lies immediately upstream of the 5' end of *Tet14*. This upstream sequence does not contain *Tet14-like*. Therefore the mouse *Tet14* and *Tet14-like* genomic loci are not as closely associated with other as are the related human genes.
A comparison of the mouse and human *Tet14* and *Tet14-Like* ORFs against the translated mouse genome has identified one retroposed *Tet14* pseudogene that contains a disrupted reading frame. Other than this pseudogene, no other homologous sequences have been identified in the mouse genome. Unlike human *TET14*, we found no evidence for the presence of *Tet14* genomic duplications in the mouse. Given the absence of other homologous sequences in the mouse and human genomes and the location of the mouse and human genes on the mouse and human X chromosomes, we believe that the human

Figure 4. Map positions of *Tet14* and *Tet14-like* on the mouse X chromosome. Map positions were determined using the T31 mouse radiation hybrid panel and the Whitehead Genome Center Auto-RHMapper program (*http://www-genome.wi.mit.edu/mouse_rh/*).

= 61.6 cR



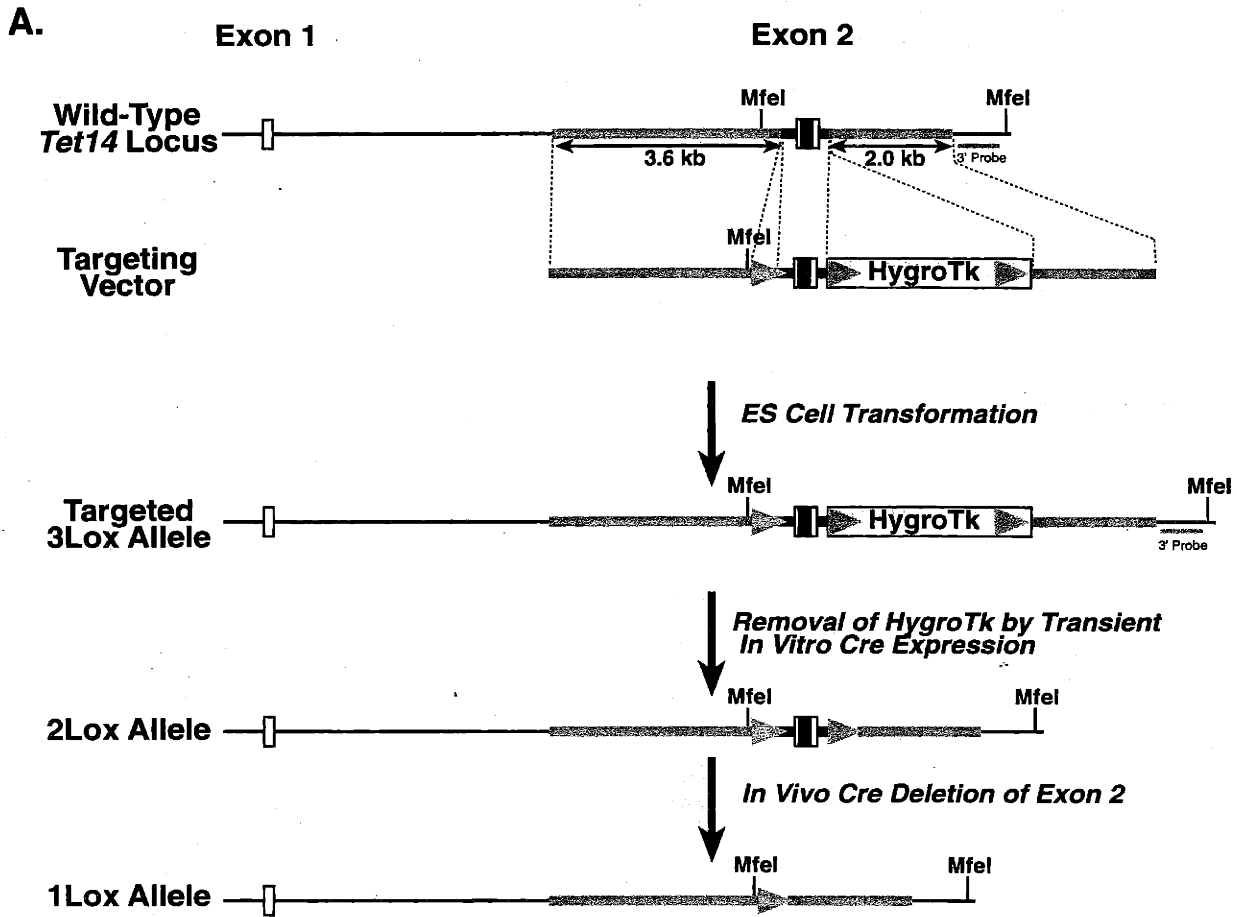
genes that we have designated as *TET14* and *TET14-Like* are, in fact, orthologs of the *Tet14* and *Tet14-like* mouse genes. However, due to the small size of the *Tet14* ORF our ability to discern distantly related genes is poor, and it is possible that other TET14 related proteins exist.

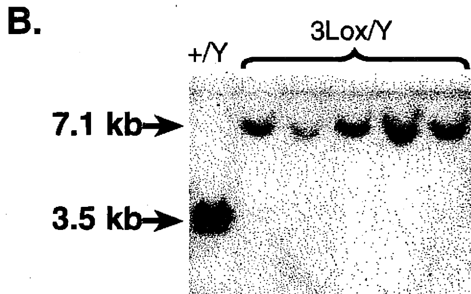
Targeted disruption of *Tet14*

Since *Tet14* is an X-linked gene that we believe may be required for testis development, deletion of *Tet14* using a conventional knockout approach with an XY ES cell line could result in a failure to achieve germline transmission. To avoid this issue, we generated a conditional knockout of the *Tet14* gene. The putative open reading frame of *Tet14* is contained entirely with the second exon. Therefore we created a "floxed" allele in which exon 2 is flanked by loxP sites (Fig. 5A). We successfully targeted the *Tet14* locus with our knockout construct and isolated multiple homologously targeted ES cell clones (Fig. 5B). We subsequently removed the HygroTk selection cassette, which itself was flanked by lox sites, by transiently transforming targeted ES cell clones with Cre recombinase. "2Lox" ES cell subclones that had lost the selection cassette, but that retained the second exon of *Tet14* and the 2 flanking loxP sites were injected into blastocysts.

Germline transmission was achieved from 2Lox ES cell subclones derived from two independently targeted ES cells (Fig. 5C). Male chimeras that transmitted the 2Lox allele were bred to Msx2-Cre female mice (Sun et al., 2000) to generate female offspring that carry the deleted "1Lox" allele of *Tet14* (Fig. 5C). We are currently breeding mature females that carry the 1Lox *Tet14* allele to wild-type males. Our analysis of the *Tet14* knockout phenotype awaits the generation of 1Lox males from these matings.

Figure 5. Genomic structure of mouse *Tet14* and targeting of the *Tet14* locus. A) The *Tet14* gene consists of two exons separated by an 8.1 kb intron. A "3Lox" targeting vector was designed in which a 685 bp fragment containing the second exon of *Tet14* was flanked by lox sites. The first loxP site was placed in the *Tet14* intron and was located 198 bps upstream of exon 2. The second and third loxP sites were part of a floxed HygroTk selection cassette that was placed 218 bps downstream of the second exon. Orange triangles represent loxP sites, and 5' and 3' homology arms are indicated in blue. B) Homologously targeted ES cell clones containing the 3Lox *Tet14* allele were confirmed by Southern blotting. Digestion with MfeI and hybridization with an external 3' probe produces a 3.5 kb band in wild-type ES cells and a 7.1 kb band in ES cells that carry a homologously targeted 3Lox allele. The HygroTk selection cassette was removed by transient expression of Cre recombinase, and the resulting 2Lox subclones were injected into blastocysts. C) Germline transmission was confirmed using a multiplex PCR assay that can distinguish wild-type, 2Lox, and 1Lox alleles of *Tet14*. Mice carrying the 2Lox allele were bred to Msx2-Cre mice to generate the 1Lox allele in which the second exon of *Tet14* has been deleted.


A.
Exon 1
Exon 2
Wild-Type
Tet14 Locus
MfeI
MfeI
3.6 kb
2.0 kb
3' Probe
Targeting
Vector
MfeI
HygroTk
ES Cell Transformation
Targeted
3Lox Allele
MfeI
HygroTk
MfeI
3' Probe
Removal of HygroTk by Transient
In Vitro Cre Expression
2Lox Allele
MfeI
MfeI
In Vivo Cre Deletion of Exon 2
1Lox Allele
MfeI
MfeI


B.
+/Y
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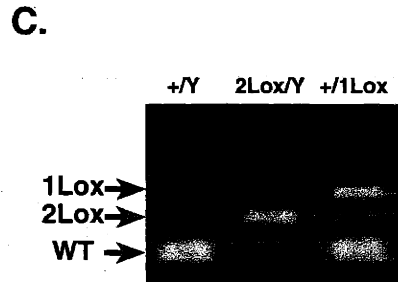
C.
+/Y
2Lox/Y
+/1Lox
1Lox
2Lox
WT

Possible Phenotypes of *Tet14* null mice

While predicting the effects of a gene knockout can be quite perilous, there is a range of phenotypes that might be expected from *Tet14* loss of function. Since the most closely related protein to TET14 appears to be the highly diverged TET14-like, we believe that functional redundancy is unlikely to mask the effects of *Tet14* loss of function. There is a remote possibility that loss of *Tet14* could result in complete XY sex-reversal. We view this as somewhat unlikely since *Tet14* is expressed slightly after the Sertoli cells begin to masculinize other cell types of the XY gonad at E11.5. It is more probable that we will observe later defects in Sertoli cell differentiation or function. The nature of these defects could include problems in formation of the testicular cords, the inability of Sertoli cells to properly interact with germ cells, or perhaps subtle defects that may be less easily defined. We expect that the differentially expressed testis and ovary genes that we have identified will be quite useful as gene expression markers if embryonic defects in testicular differentiation are exhibited.

Materials and Methods

Sequencing of the *Tet14* mouse locus

The RPCI-22 mouse Bac library (Research Genetics) was screened with a Tet14 cDNA fragment, and one *Tet14* positive Bac clone (RPCI-530_D_11) was identified. Initially, a combination of Bac subclones and long distance PCR products were generated from this clone to obtain sequence from the *Tet14* locus. Sequence gaps were then filled by direct Bac sequencing.

Generation of targeted ES cells

We constructed a 3Lox *Tet14* targeting construct using PCR products amplified with Advantage HF2 polymerase (Clontech). All PCR products were sequenced to ensure the absence of point mutations. The final 3Lox targeting construct contained a 5' homology arm of 3.6 kb derived from the *Tet14* intron followed by a loxP site and a 685 bp fragment that contained the second exon of *Tet14.* A floxed HygroTk selection cassette was placed 3' of these sequences, followed by a 2.0 kb 3' homology arm. The v6.5 ES cell line (Rideout et al., 2000) was electroporated with 40 μg of linearized *Tet14* 3Lox targeting construct and selection was performed with 350 μg/ml Hygromycin B (Roche). 456 ES cell colonies were picked and screened by long distance PCR assay that was specific to clones that are correctly targeted at the 5' arm. Eleven positive clones were identified, and the 3' targeting of all eleven clones was confirmed by Southern blot analysis. The HygroTk selection cassette was removed by transient transfection with a Cre expression plasmid and negative selection in 2 μm Ganciclovir (Sigma). 2Lox subclones that had lost the selection cassette but that retained the second exon of *Tet14* were identified by PCR. 2Lox ES cell subclones were injected into Balb/c or C57Bl/6 blastocysts and transferred to pseudopregnant Swiss Webster females. Chimeras that transmitted the 2Lox allele were bred to *Msx2-Cre* (Sun et al., 2000) females to generate mice carrying the deleted 1Lox allele.

Acknowledgements

We thank Rudolf Jaenisch for the V6.5 ES cell line and Msx2-Cre mice, and Alex Bortvin and members of the Jaenisch lab for ES cell targeting reagents and advice.

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Chapter 7

Conclusions and Future Directions

Gonadal sex differentiation of mammals is a complex process that involves the interaction, differentiation, and organization of multiple cell types. Progress in this field has been limited, in part, due to the small number of gene expression differences between the differentiating embryonic ovary and testis that have been identified. In the work presented here I have significantly expanded upon the known sexually dimorphic gene expression differences that occur during gonadal differentiation in the mouse. Two separate cDNA subtractions were performed in which fourteen genes that are upregulated during testis differentiation and two genes that are upregulated during ovary differentiation were identified.

Whole-mount *in situ* hybridization revealed that these genes are expressed predominantly in different somatic cell types of the testis and ovary. Moreover, gene markers of Sertoli and other testis cell lineages are upregulated in a "center-to-pole" pattern in which expression is initiated in central regions and later spreads to the anterior and posterior poles of XY gonads. In a complementary fashion ovarian genes are initially upregulated at the anterior and posterior poles of XY gonads and suppressed in central regions. At later time points the expression of ovarian genes is extinguished at the poles of XY gonads as well.

In the embryonic ovary we observed center-to-pole and anterior-posterior waves of somatic expression. However, even more striking than the somatic expression patterns is the anterior-posterior wave of *Stra8* expression that occurs in female germ cells. This expression is remarkable both in the clarity of the anterior-posterior wave and in the extended length of its duration, approximately four days. This pattern of expression,

which is also observed with the meiotic *Dmc1* gene, has revealed the hitherto unknown fact that embryonic ovarian germ cells differentiate in an anterior-posterior wave.

The final portion of my thesis describes the targeted disruption of *Stra8* and *Tet14*, a novel X-linked gene that is expressed in embryonic and adult testis. While these lines of functional analysis are works in progress, the initial phenotype data available for *Stra8* indicates that this knockout disrupts oocyte development. The complete characterization of the *Stra8* null phenotype may provide valuable insights into early events in meiosis and possibly even female germ cell sex determination.

Future Directions

Waves of Differentiation

One of the more interesting and more difficult problems to address is the identification of the regulatory components that control the waves of expression that I have documented. With regards to the center-to-pole wave that occurs during the early stages of testis differentiation, it seems apparent that it is the expression of *Sry* in a center-to-pole pattern that causes other testicular genes to be upregulated similarly. Therefore, the real question may be "what causes *Sry* to be expressed in a center-to-pole pattern?" The identification of the genes responsible for XY^{DOM} sex-reversal may be informative in this regard. The *Sry* allele carried on the Y^{DOM} chromosome is thought to be misexpressed when bred onto a C57Bl/6 strain background due to incompatibilities between trans acting factors and cis regulatory elements on the Y^{DOM} chromosome (Albrecht and Eicher, 1997; Nagamine et al., 1999). Three loci that may be involved in this incompatibility have previously been mapped to mouse chromosomes 2, 4, and 5

(Eicher et al., 1996). If these genes participate in the regulation of *Sry*, then their identification may provide some insight into the center-to-pole wave of expression. The task of finding these genes is not trivial given that multiple loci are involved and the contribution of these loci to the sex-reversal effect is not equal. Therefore, a traditional positional cloning approach may not be practical. However, since most inbred strains of mice do not exhibit XY^{DOM} sex-reversal, the use of polymorphisms to identify regions of the mouse genome that differ between C57Bl/6 and other strains of mice may be effective in narrowing the candidate regions on chromosomes 2, 4, and 5.

Waves of expression were also detected during ovary development. While I did observe a center-to-pole expression pattern for *follistatin*, the more impressive patterns were the anterior-posterior waves produced by the somatically expressed *Adamts-19* gene and the germ cell expressed *Stra8* gene. It will be important to find more anterior-posterior differences in gene expression to understand this phenomenon and its role in ovarian differentiation. One obvious approach would be to isolate embryonic ovaries at E11.5, E12.5, or E13.5 and to separate the anterior 1/3 and the posterior 1/3 away from each other for expression analysis. A combination of cDNA array experiments and cDNA subtractions should yield more anterior-posterior differences. In addition to the anterior-posterior upregulation of genes this should also identify genes that demonstrate an anterior-posterior loss of expression. *Stra8* actually falls into both of these categories as it is initial upregulated in an anterior-posterior wave and then it is subsequently down regulated in a similar pattern. This approach may also reveal a complementary posterior-anterior wave of upregulation. It may also be informative to determine whether the

anterior-posterior expression of somatic and germ cell genes is perturbed in the *follistatin* and *Wnt4* knockout mice that have been generated by other labs.

***Stra8* and germ cell differentiation**

I initially approached the anterior-to-posterior wave of *Stra8* expression in embryonic ovaries from the perspective of germ cell sex differentiation rather than purely as a marker of meiotic entry. Clearly these two issues are closely tied to each other. Mouse germ cells that enter meiosis embryonically differentiate as female, while germ cells that arrest in a testicular environment differentiate as male and enter meiosis postnatally. One central question regarding germ cell sex differentiation is how closely linked are the commitment to female development and meiotic entry.

Work by McLaren and colleagues has demonstrated that between E12.5 and E13.5 ovarian germs lose their capacity to respond to a testicular environment in tissue culture. *Stra8* is expressed beginning at E12.5, but the majority of ovarian germ cells do not express *Stra8* until sometime between E13.5 and E14.5. What is the significance of the fairly sudden transition that McLaren has observed and the more gradual appearance of *Stra8* positive germ cells? One possibility is that germ cells may commit to the female pathway and then wait for additional developmental cues to enter meiosis. However, it seems just as likely that the apparent sudden transition that McLaren saw is an experimental artifact. The experiment required the disaggregation of embryonic testes and their subsequent reaggregation with isolated female germ cells. Since it takes some time for these cells to recover, this delay could prevent the majority of non-meiotic E13.5 germ cells from responding to the testicular environment even if in a non-perturbed environment they would normally have the capacity to develop as male. It may take the

identification of the factors that control the anterior-to-posterior differentiation of female germ cells for us to completely understand the relationship between meiotic entry and female sex determination.

Functional Analysis of testis and ovary genes

Gene knockouts of *Stra8* and a novel testis gene, *Tet14*, have been generated and the characterization of these knockouts is in progress. In addition, the targeted disruption of the ovarian gene *Adamts19* is underway. It will be quite interesting to see what role this secreted metalloprotease plays in ovary organogenesis. While it is currently not practical to generate knockouts for every gene that has a sexually dimorphic expression pattern, an alternative may be to test gene function using an organ culture system. Morpholino antisense oligonucleotides have recently been used in organ culture to successfully knock-down the expression of genes in the genital ridges of rat embryos (Roberts et al., 2002; Visser et al., 2001). Using this technique one could screen many candidate genes for loss of function effects. For genes that are expressed in multiple tissues, this approach would have the additional advantage that pleiotropic effects on other aspects of development would not interfere with the observation of gonadal defects. Organ culture can also be used to observe the effects of exogenously added factors (Adams and McLaren, 2002; Livera et al., 2000; Morita and Tilly, 1999). For instance, follistatin protein is commercially available (Sigma), so the effects of follistatin on embryonic testis development could be experimental examined. Similarly, the bioactive hexadecapeptide derived from the proteolytic processing of CBLN1 is also commercially available (American Peptide Company, Inc.). Normally *Cbln1* expression is upregulated during embryonic testis differentiation and down-regulated in embryonic ovaries. The

effects of the CBLN1 peptide on ovary development could be assessed by introducing the peptide to embryonic ovaries cultured *in vitro*.

Loss of function experiments can be very informative, especially with the range of different testicular and ovarian molecular markers that are now available to examine mutant gonadal phenotypes. However, to determine how a protein is functioning often requires a different class of experiments. These experiments include procedures such as immunolocalization to determine where the protein is in the cell and immunoprecipitation or yeast two-hybrid to reveal protein-protein interactions. These experiments have recently been initiated for the STRA8 protein and may help us to interpret the results of STRA8 loss of function in germ cells.

The ultimate objective is to understand how sex differentiation of the somatic and germ cell lineages is accomplished, what molecules participate in this process, and how these molecules function to attain this end. The work that I have presented here is a small step in this direction and provides multiple avenues by which different aspects of this developmental problem can be approached.

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Appendix

Expression of *DAZ*, an *Azoospermia Factor* Candidate, in Human Spermatogonia

Douglas B. Menke, George L. Mutter, and David C. Page

Rossel M, Schuffenecker I, Schlumberger M, Bonnardel C, Modigliani E, Gardet P, Navarro J, et al (1995) Detection of a germline mutation at codon 918 of the *RET* proto-oncogene in French MEN 2B families. Hum Genet 95:403–406

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Expression of *DAZ,* an *Azoospermia Factor* Candidate, in Human Spermatogonia

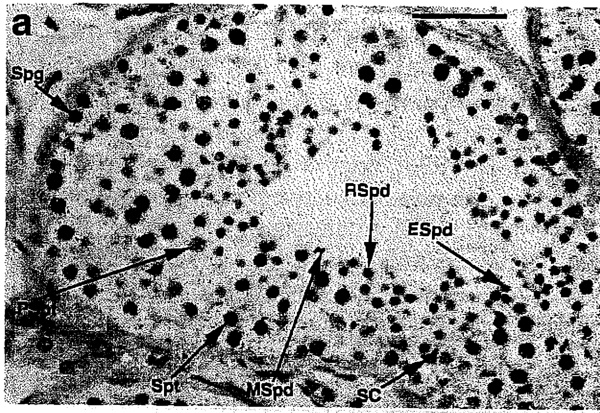
To the Editor:

Three percent to 4% of men have severe defects in sperm production that result in infertility (van Zyl et al. 1975; Hull et al. 1985). Most of these men are otherwise healthy, and the cause of their spermatogenic failure is rarely identified with certainty. Little is known about possible contributions from genetic factors, but some cases may be due to mutations that disrupt male germ-cell development without affecting the soma. Such "pure male sterile" genes have been identified in invertebrates (Ellis and Kimble 1994; Castrillan et al. 1993). In humans, no pure sterile factor—male or female—has been unequivocally defined at the biochemical level, although some critical genetic loci have been mapped.

The *azoospermia factor* (*AZF*), found on the long arm of the Y chromosome, may be the most thoroughly studied pure male sterile locus in humans. In 1976, Tiepolo and Zuffardi reported microscopic deletions of Yq in six men with azoospermia (no sperm in semen). On the basis of these findings, they proposed that Yq carries an *AZF* gene or gene complex required for spermatogenesis (Tiepolo and Zuffardi 1976). Recently, Tiepolo and Zuffardi's hypothesis has received strong experimental support (Ma et al. 1992; Kobayashi et al. 1994; Najmabadi et al. 1996; Vogt et al. 1996). In particular, 13% of men with nonobstructive azoospermia were found to have deletions of a consistent, specific portion of Yq—an "*AZF* region" (Reijo et al. 1995). The deletions were not present in the affected individuals' fathers but had arisen de novo, establishing that the deletions were the cause of azoospermia rather than an incidental finding.

De novo deletions of this *AZF* region have been found to result in a wide range of spermatogenic defects. In some *AZF*-deleted individuals, testis biopsies revealed the complete absence of germ cells ("Sertoli cell–only syndrome"). In other individuals, early spermatogenic cells were observed ("testicular maturation arrest"). In two of the latter individuals, spermatogenesis had sometimes progressed through meiosis to the stage of condensed spermatids (Reijo et al. 1995). Indeed, we have shown recently that deletion of *AZF* is compatible with completion of spermatogenesis, albeit at greatly reduced output: two men with severe oligozoospermia (markedly reduced but nonzero semen sperm counts) were found to have de novo, *AZF*-region deletions similar or identical to those found in unrelated azoospermic men (Reijo et al. 1996*a*). Thus, spermatogenesis can, in some instances, proceed without *AZF*. However, spermatogenic output is severely diminished in the absence of *AZF,* and in some cases germ cells (even spermatogonial stem cells) are found to be completely lacking. As we and our colleagues have suggested, this wide array of *AZF* phenotypes might be explained most simply by a defect (with variable expressivity) in spermatogonia, the stem cells that are the source of the spermatogenic lineages in the adult testis (Reijo et al. 1995, 1996*a*).

The molecular identity of *AZF* is not yet known with certainty, but much experimental evidence has accumulated in favor of *DAZ* (*deleted in azoospermia*), a multicopy gene cluster located in this *AZF* region (Reijo et al. 1995; Saxena et al. 1996). As revealed by northern blotting of human tissue RNAs, *DAZ* is transcribed specifically in testis. On the basis of sequence comparisons, *DAZ* appears to encode an RNA-binding protein. Although no human *DAZ* point mutants have been reported, recent genetic findings in *Drosophila* provide strong support for the hypothesis that *DAZ* is *AZF.* Eberhart and colleagues have characterized a *Drosophila* gene, *boule,* whose product shows striking amino acid similarity to the human DAZ protein, especially in the RNA-binding domain. Just as with human *AZF,* loss-of-function mutations in *Drosophila boule* disrupt spermatogenesis and result in azoospermia but spare the soma (Eberhart et al. 1996). Thus, the *Drosophila* ho-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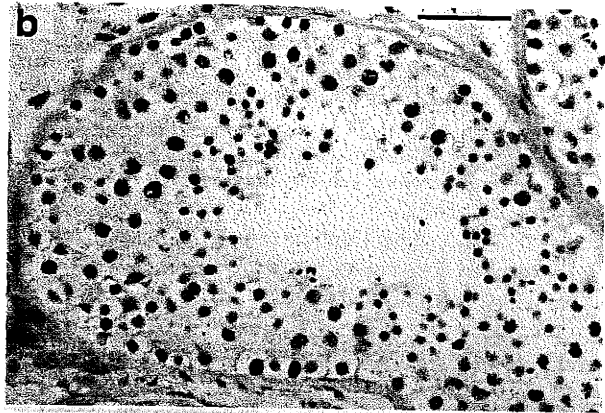
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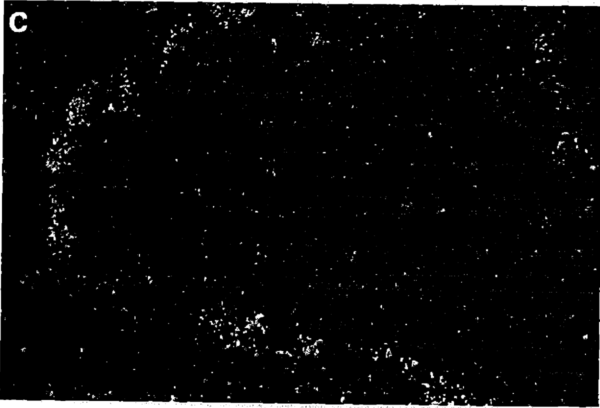
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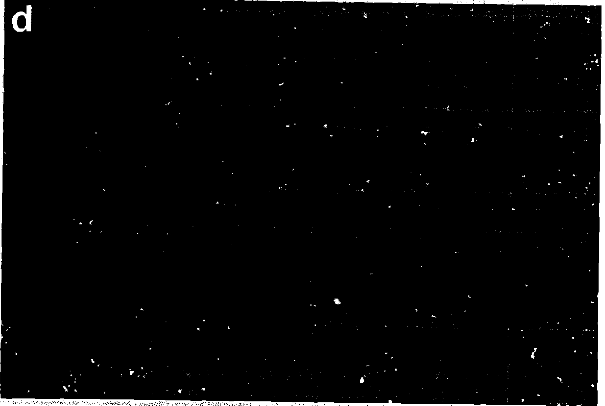
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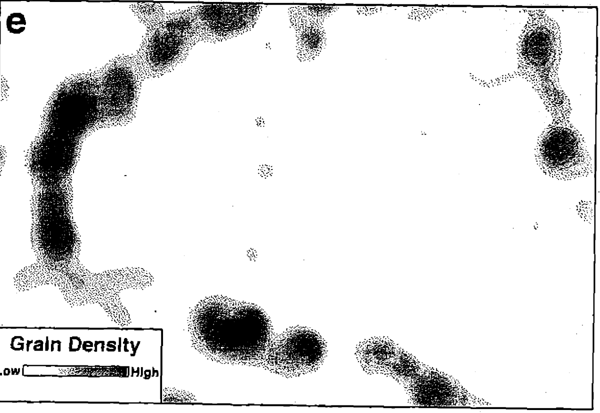
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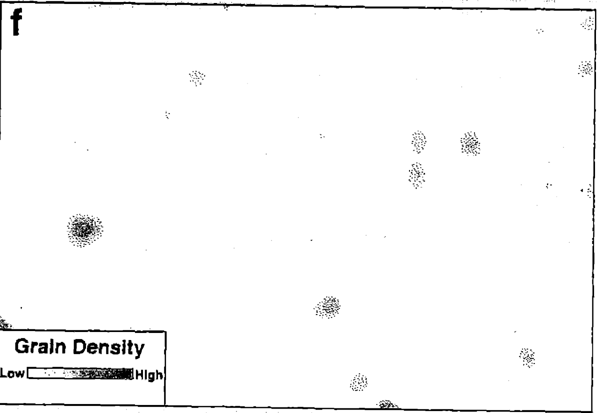
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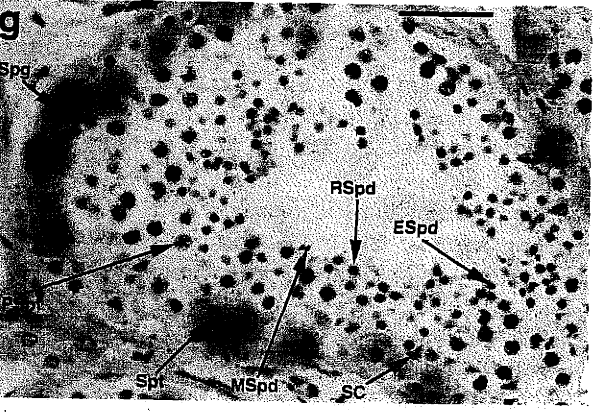
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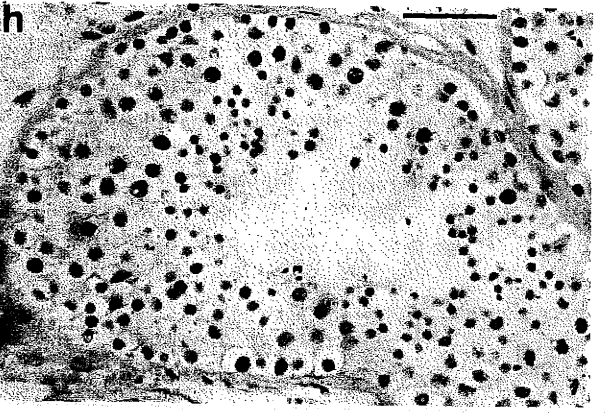
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mologue of *DAZ* is a pure male sterile gene with striking parallels to *AZF*.

Like *Drosophila boule,* human *DAZ* is expressed specifically in the testis (Reijo et al. 1995; Eberhart et al. 1996). Given the prospect that *DAZ* might be *AZF,* we set out to study its expression in the human testis in detail. We were interested in determining (i) whether *DAZ* is expressed in the somatic cells of the testis, in germ cells, or both and (ii) whether the pattern of *DAZ* expression would be consistent with the array of spermatogenic defects observed in men with deletions of *AZF*.

To localize *DAZ* transcripts, radio-labeled oligonucleotide probes were hybridized to sections of normal human adult testes, essentially as described by Mutter and colleagues (1993). Testes were obtained from the Department of Pathology, Brigham and Women's Hospital, Boston, following institutional review board approval. The tissues originated from three men with prostate cancer who had undergone elective orchiectomies. Tissues were fixed in 4% paraformaldehyde and embedded in paraffin. All testes appeared histologically normal, with active spermatogenesis. Five-micron sections were cut from paraffin-embedded testes, dewaxed in xylene, and rehydrated in graded ethanols to water. Two synthetic antisense probes, each 50 nt in length, were selected from the published *DAZ* cDNA sequence (Reijo et al. 1995). Probe A (GGCAACTGATATCCAGTG-GTGACCTGAAATGGTGAACTTGGATAAGCAGG) was chosen from within a 72-bp segment that is tandemly repeated seven times in the *DAZ* coding sequence. Probe B (CTTCGGTCCACAGATTTCTCCTTTGCT-CCCCAACAGGGCACTGCGGTGGC) was also chosen from coding sequence, but outside the tandem repeats. Oligonucleotides complementary to probes A and B were used as negative controls. All four probes were purified on acrylamide gels and then end-labeled with ^{35}S-α-dATP by use of terminal deoxynucleotidyl transferase. Hybridizations were carried out overnight in a humidified chamber at 42°C using a solution of 4 × SSC (1 × SSC = 0.15 M NaCl, 15 mM sodium citrate [pH 7.4]), 50% formamide, 1 × Denhardt's (0.02% Ficoll 400, 0.02% polyvinyl pyrrolidone, 0.02% BSA) 1% sarcosyl, 0.2 M sodium phosphate (pH 7.0), 10% dextran sulfate, 250 μg/ml yeast tRNA, 500 μg/ml salmon sperm DNA, 200 mM DTT, and 10^6 cpm/100 μl of ^{35}S-α-dATP end-labeled oligonucleotide. Sections were then washed four times for 15 min each in 1 × SSC at 55°C. After drying, slides were dipped in 50% NTB-2 emulsion (Kodak diluted with water), stored at 4°C for 10–176 d, and developed. Images of autoradiographic grain density were electronically processed with commercial software (Adobe Photoshop). In brief, conventional dark-field images were inverted and brightness levels adjusted so that autoradiographic silver grains appeared black on a white background. This inverted dark-field image was then subjected to "Gaussian-blur" filtering, by which signal intensities in individual pixels are locally averaged so as to produce continuous intensity contours.

Lengthy exposures were required for optimal signal detection: 87 d for probe A and 176 d for probe B. The signal-to-noise ratio obtained with probe A, derived from a seven-fold tandem repeat within the *DAZ* transcript, was somewhat better than that obtained when probe B was used, derived from a nonrepeated portion of the transcript. However, both probes revealed a similar distribution of *DAZ* transcripts, and multiple exposures on testis sections from three men yielded consistent results. As shown in figure 1, using probe A, transcripts were detected just inside the perimeter of seminiferous tubules—not around the entire circumference of the tubule, but in discrete patches (similar results obtained with probe B; not shown). Image processing (fig. 1*e, f*) facilitated identification and classification of individual cells underlying areas of intense signal. Spermatogonia were clearly the source of most of the intense signals. Occasional early (leptotene/zygotene) primary spermatocytes had similarly high levels of transcripts, but transcripts were not detected in later (pachytene) primary spermatocytes with either probe. Neither probe revealed the presence of *DAZ* transcripts in other spermatogenic cells or in the somatic cells of the testis (e.g., Sertoli, Leydig, peritubular cells).

Our findings with human Y-chromosomal *DAZ*—that it appears to be expressed exclusively in germ cells and most abundantly in spermatogonia (fig. 1)—may extend to autosomal homologues found in humans and mice. In both humans and mice, there exists an autosomal *DAZ* homologue (*DAZH* in humans; *Dazh*/*Dazla* in mice) that is expressed in testes and, at a much lower level, in ovaries (Cooke et al. 1996; Reijo et al. 1996*b;* Saxena et al., in press). The absence of *Dazh* transcripts

Figure 1 Hybridization of *DAZ* probe to human adult testis. In the left column (panels *a, c, e,* and *g*) are images of a seminiferous tubule probed with antisense probe A. In the *right* column (panels *b, d, f,* and *h*) are images of an adjacent tissue section probed with the complementary sense probe. *a* and *b,* Bright-field photographs, sections stained with hematoxylin and eosin; autoradiographic grains dimly visible in background. *c* and *d,* Dark-field photographs of the same field, highlighting autoradiographic silver grains. *e* and *f,* Autoradiographic grain density contours produced by subjecting dark-field images (panels *c* and *d*) to Gaussian blur filtering (see text). *g* and *h,* Grain-density contours (panels *e* and *f*) overlaid onto bright-field histology (panels *a* and *b*). Cell types: Spg = spermatogonium; Spt = leptotene/zygotine primary spermatocyte; PSpt = pachytene primary spermatocyte; RSpd = round spermatid; ESpd = elongating spermatid; MSpd = mature spermatid; and SC = Sertoli cell. All images at 400× magnification; bar = 50 μm.

in germ-cell–deficient mice suggests that expression of this autosomal gene is restricted to germ cells in both testes and ovaries (Reijo et al. 1996*b;* J. Seligman, R. Reijo, D. C. Page, unpublished data). Specifically, autosomal mouse *Dazh* appears to be transcribed in spermatogonia, as indicated by developmental northern-blotting studies (Reijo et al. 1996*b*)—like human Y-chromosomal *DAZ.*

A portion of the testis in situ signal we observed with *DAZ* probes may have originated from *DAZH.* If so, then the specificity of the observed hybridization pattern (fig. 1) suggests that it may describe the expression of both Y-chromosomal *DAZ* and its autosomal homologue.

Although many mammalian genes are expressed in the testis, *DAZ*'s restricted pattern of expression is unusual. Numerous genes are expressed exclusively in male germ cells (e.g., protamines and testis-specific isoforms of tubulins, histones, and lactate dehydrogenase), mostly in meiotic or postmeiotic cells, but not in the earliest stages of the spermatogenic pathway (Distel et al. 1984; Drabent et al. 1991; Salehi-Ashtiani and Goldberg 1993; Wykes et al. 1995). Other genes are expressed in premeiotic germ cells (e.g., c-myc, c-kit, fos, jun, MAGE -1 and -4, hsp60, and FSH β), but also in one or more somatic tissues (Koji et al. 1988; Manova et al. 1990; Kurata et al. 1993; Markkula et al. 1995; Meinhardt et al. 1995; Takahashi et al. 1995). The specificity of *DAZ* expression in spermatogonia and their immediate derivatives, early primary spermatocytes, suggests that human *DAZ* functions in the first phases of spermatogenic differentiation—or earlier, in the maintenance of the spermatogonial stem-cell populations.

As we have pointed out elsewhere (Reijo et al. 1995), the phenotypes associated with deletions of *AZF* suggest that it may function in spermatogonia—where we have now shown *DAZ* to be expressed. Additional evidence that *DAZ* may be *AZF* is found in *Drosophila boule,* a homologue of *DAZ* and, like *AZF,* a pure male sterile locus. The products of the human *DAZ* and *Drosophila boule* genes exhibit remarkable amino acid sequence similarity (Reijo et al. 1995; Eberhart et al. 1996). We have now established that the human and *Drosophila* genes also have similar expression patterns: both are transcribed exclusively in male germ cells.

In addition to providing evidence for the molecular identity of *AZF,* our findings suggest possible avenues for future studies of spermatogonia. Spermatogonia play a central role in human heredity, genetic disease, and evolution. They are the source of all male gametes and, as some have suggested, may be the cells in which most germ-line mutations arise (Haldane 1947; Ketterling et al. 1993; Shimmin et al. 1993). Given these observations, it is remarkable how little is known about the molecular biology of this stem-cell population (Hecht 1995). The availability of molecular markers like *DAZ* may help remedy this situation.

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