Sry induces cell proliferation in the mouse gonad

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SUMMARY

Sry is the only gene on the Y chromosome that is required for testis formation in mammals. One of the earliest morphological changes that occurs as a result of *Sry* expression is a size increase of the rudimentary XY gonad relative to the XX gonad. Using 5′-bromo-2′-deoxyuridine (BrdU) incorporation to label dividing cells, we found that the size increase corresponds with a dramatic increase in somatic cell proliferation in XY gonads, which is not detected in XX gonads. This male-specific proliferation was observed initially in the cells of the coelomic epithelium and occurred in two distinct stages. During the first stage, proliferation in the XY gonad was observed largely in SF1-positive cells and contributed to the Sertoli cell population. During the second stage, proliferation was observed in SF1-negative cells at and below the coelomic epithelium and did not give rise to Sertoli cells. Both stages of proliferation were dependent on *Sry* and independent of any other genetic differences between male and female gonads, such as X chromosome dosage or other genes on the Y chromosome. The increase in cell proliferation began less than 24 hours after the onset of *Sry* expression, before the establishment of male-specific gene expression patterns, and before the appearance of any other known male-specific morphological changes in the XY gonad. Therefore, an increase in cell proliferation in the male coelomic epithelium is the earliest identified effect of *Sry* expression.

Key words: Proliferation, Organogenesis, Testis, Sex determination, Sertoli cell, *Sry*

INTRODUCTION

Gonad development is a unique system in which a single rudimentary tissue can be induced to form one of two different organs, the ovary or the testis. In mammals, the choice between these two fates is initiated by a single gene on the Y chromosome, *Sry* (sex determining region of the Y chromosome). If *Sry* is expressed in the rudimentary gonad, either from the Y chromosome or from an ectopic transgene, a testis forms (Gubbay et al., 1990; Koopman et al., 1991; Eicher et al., 1995). If *Sry* is not expressed, as in XX individuals or in cases where *Sry* is mutated or deleted, an ovary forms (Lovell-Badge and Robertson, 1990; Page et al., 1990; Hawkins et al., 1992). Therefore, *Sry* is considered a genetic switch that directs the identity of a bipotential organ. *Sry* encodes a DNA binding protein believed to initiate the male pathway and/or inhibit the female pathway by regulating the transcription of downstream genes (Eicher and Washburn, 1986; Harley et al., 1992; Lovell-Badge, 1993, Rimini et al., 1995). However, no direct molecular targets of SRY have been identified, and the mechanisms by which *Sry* specifies the male pathway and remodels the rudimentary gonad are largely unknown.

The functional transcript of *Sry* is expressed in the mouse gonad during a narrow window of development, between 10.5 and 12.5 days post coitum (dpc) (Hacker et al., 1995). By 12.5 dpc, clear morphological changes are apparent in the XY gonad that do not occur in the XX gonad. One of the earliest of these changes is a doubling of the size of the XY gonad, relative to the XX gonad. This male-specific size increase at the earliest stages of sex determination has been documented in the gonads of a variety of mammals, including mice, rats, rabbits, and humans (Mittwoch et al., 1969; Buehr and Petzoldt, 1974; Mittwoch and Mahadevaiah, 1980; Hunt and Mittwoch, 1987). Prior to the discovery of *Sry*, it was hypothesized that this increase in the growth rate of XY gonads triggered male sex determination (Mittwoch, 1986). We investigated the possibility that *Sry* initiates a cellular pathway responsible for this size increase as an early step in testis organogenesis.

Several known mechanisms contribute to the size increase of the XY gonad. Organ culture experiments show that XY gonads recruit cells from the mesonephros (Buehr et al., 1993; Martineau et al., 1997; Merchant-Larios and Moreno-Mendoza, 1998), and observations of testicular cells in electron micrographs indicate that Sertoli cells increase in size as they differentiate (Magre and Jost, 1991). In addition, many studies have considered the contribution of somatic cell proliferation to the gonads of both sexes. In 1951, Witschi suggested that the testis is derived from proliferation of cells that originate from the adjacent mesonephros, while the ovary is derived from proliferation of a layer of coelomic epithelial cells that cover the coelomic surface of the gonad. Based on observations of a thickening coelomic epithelium, earlier microscopists

proposed that the gonad of both sexes is derived from proliferation in this region (Whitehead, 1904; Brambell, 1927; Torrey, 1945). In 1979, Merchant-Larios proposed that the testis arises from the proliferation of cells in both the internal mesenchyme of the gonad and the coelomic epithelium, while the ovary arises largely from the coelomic epithelium. Scanning electron micrographs (Capel and Lovell-Badge, 1993) and DiI labeling of single cells in cultured gonads (Karl and Capel, 1998) also support an important role for the coelomic epithelium as a contributor of somatic cells to both ovaries and testes. Discrepancies in the classic theories surrounding proliferation and the origin of somatic cells in the gonad may be due to several factors, including difficulties in identifying proliferating cells, the lack of compatible markers to identify cell types, and the inability to chromosomally sex undifferentiated gonads. In this study, incorporation of 5′-bromo-2′-deoxyuridine (BrdU) into the DNA of dividing cells was used to compare the pattern of proliferation in chromosomally sexed XY and XX gonads. We determined that *Sry* initiated a dramatic increase in somatic cell proliferation at the coelomic epithelium of XY gonads at 11.25 dpc. In contrast, proliferation in the female gonad changed very little at this time.

MATERIALS AND METHODS

Staging, dissecting and genotyping prenatal gonads

Timed matings were produced by housing CD-1 or C57BL/6J (B6) female mice with males of the same strain overnight, or by housing B6 females with C57BL/6J-Y^{POS} TgN(*Sry*)4Ei males. C57BL/6J-Y^{POS} TgN(*Sry*)4Ei males carry both a Y chromosome from *Mus domesticus poschiavinus* mice (Y^{POS}) and a multicopy *Sry* transgene (*TgN (Sry)4Ei*) derived from *Mus musculus* (Eicher et al., 1995).

Fetuses were dissected between 10.5 and 12.5 days post coitum (dpc). For more accurate staging, the tail somite stage (ts) of the embryo was determined by counting the number of somites in the tail, beginning at the first somite posterior to the hind limb. Using this method, 10.5 dpc corresponds to approximately 8 ts, 11.5 dpc to 18 ts, and 12.5 dpc to 30 ts (Hacker et al., 1995). To determine the sex of each fetus, the amnion was collected and stained to identify sex chromatin bodies in XX cells (Palmer and Burgoyne, 1991a). The genotypes of embryos carrying Y^{POS} and/or the *Sry* transgene were determined by PCR, using primers to detect the Y chromosome, the *Sry* transgene, and myogenin as a control (Capel et al., 1999).

BrdU labeling, tissue processing and immunohistochemistry

To label dividing cells, pregnant mice received a single intraperitoneal (i.p.) injection of BrdU (Sigma B-5002), using 50 mg BrdU per kg of
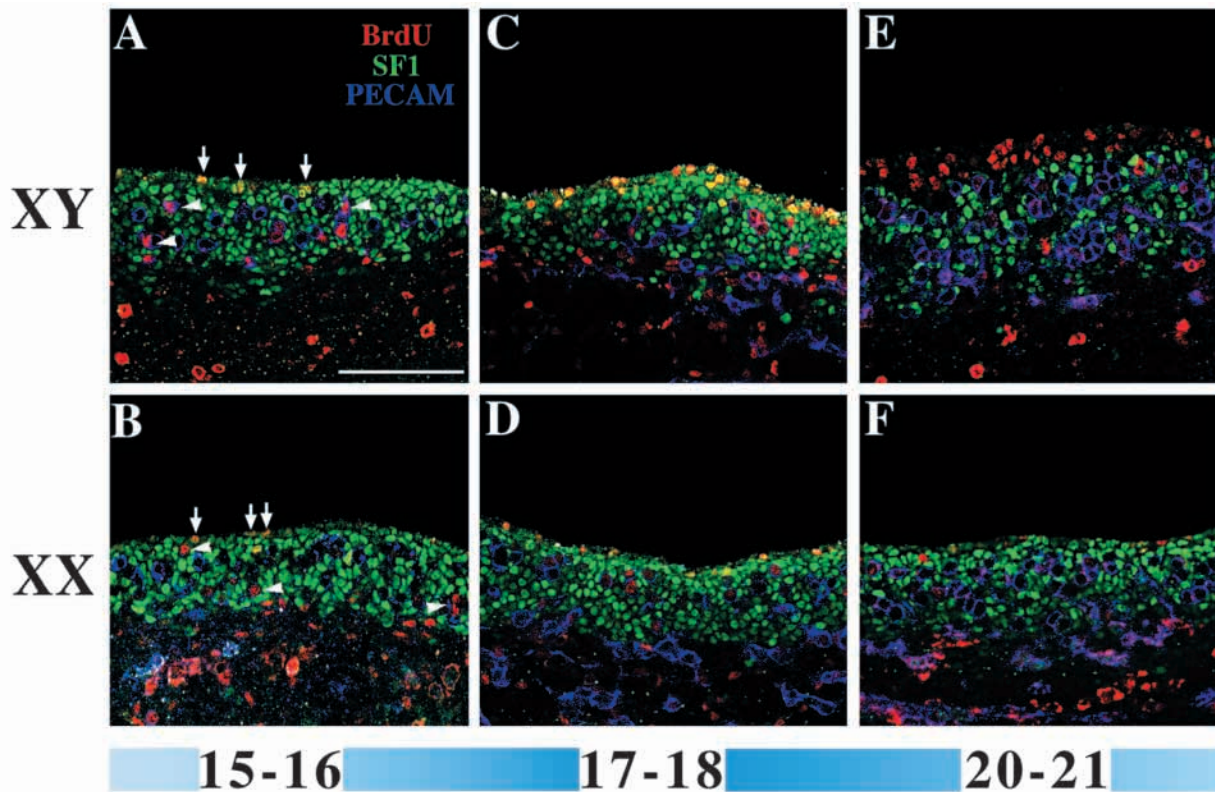
Fig. 1. Confocal images comparing cell proliferation in CD1 XY and XX gonads between 15 and 21 ts. BrdU (red) and SF1 (green) are nuclear labels, therefore, dividing SF1-positive cells are yellow (arrows in A and B). PECAM (blue) is a membrane label for germ cells and vasculature, which proliferate in both sexes at all stages shown (red label surrounded by blue, arrowheads in A and B). (A,B) Somatic cell proliferation between 15-16 ts is indistiguishable between sexes, and is concentrated at or near the coelomic epithelium. (C,D) By 17-18 ts, the first difference between XY and XX gonads is visible as an increase in the number of SF1-positive cells proliferating at the coelomic epithelium. (E,F) By 20-21 ts, proliferation in the XY gonad far exceeds proliferation in the XX gonad and is still concentrated at and near the coelomic epithelium, but is observed mainly in SF1-negative cells (red, E). In all panels, the coelomic epithelium is oriented at the top of the frame and the mesonephros is toward the bottom. The shading of the blue bar approximates the percentage of maximum *Sry* expression detected at each stage in XY gonads using RNAse protection (modeled after Hacker et al., 1995). Non-nuclear BrdU background is occasionally visible in germ cells and red blood cells. Scale bar, 100 μm.

body weight. BrdU is an analog of thymidine, and is incorporated into DNA during S phase (Dolbeare, 1995). Two hours after injection, pregnant females were killed. The gonads were removed from each fetus and fixed overnight in 2% paraformaldehyde in PBS at 4°C.

Gonads from mice of the same strain and genotype were pooled at specific stages and processed as whole mounts. BrdU was detected using procedures modified from Schutte et al. (1987) and Carayon and Boyd (1992). Samples were rinsed three times with PBS, then rinsed once in 0.01N HCL for 3 minutes. Samples were incubated in 1 mg/ml pepsin (Sigma P-6887) in 0.01 N HCL for 1 hour at 37°C, rinsed thoroughly 3 times in reaction buffer (0.1 M Tris pH 7.5, 50 mM NaCl, 10 mM MgCl$_2$), about 3 minutes per wash, incubated for 1 hour at 37°C in 100 U/ml DNAse I (Sigma DN-25) in reaction buffer, then for one hour at room temperature in 10% goat serum, 0.1% Triton X-100 in PBS. Samples were incubated overnight in primary antibodies diluted in 1% goat serum, 0.01% Triton X-100 in PBS at 4°C, rinsed 3 times in PBS, then incubated in secondary antibodies diluted 1:500 in 1% goat serum, 0.01% Triton X-100 in PBS for 2 hours at room temperature.

BrdU was detected using a mouse monoclonal IgG antibody (Boehringer Mannheim, clone BMC 9318) diluted 1:50. Steroidogenic factor 1 (SF1) was detected using a 1:100 dilution of a rabbit polyclonal antibody generously donated by Ken-ichirou Morohashi. Germ cells and vasculature were identified using a 1:100 dilution of a rat IgG antibody against mouse platelet endothelial cell adhesion molecule (PECAM; from Pharmingen 01951D). The basal lamina surrounding the testis cords was detected with a 1:50 dilution

of a rabbit polyclonal antibody against laminin-1 generously donated by Harold Erickson. After incubation in the appropriate secondary antibodies (Jackson Immunologicals), samples were rinsed 3 times in PBS and mounted for confocal imaging as described in Karl and Capel (1998).

BrdU pulse/chase procedure

In pulse/chase experiments, pregnant females received an i.p. injection of 50 mg/kg of BrdU followed by an i.p. injection of 50 mg of thymidine one hour later. In the presence of excess thymidine, BrdU is no longer incorporated into DNA (Haaf, 1996). After the thymidine chase, gestation was allowed to continue either for 3 hours, or until embryonic development reached 12.8 dpc (approximately 33 ts). Gonads dissected at 3 hours were processed according to previously described whole-mount protocols. Gonads collected at 33 ts were embedded in 20% PBS:OCT and cryosectioned as described in Karl and Capel, 1998, before BrdU was detected. Using this pulse/chase technique, cells in S phase during the hour of the BrdU pulse incorporate BrdU into their DNA. In subsequent divisions, this labeled DNA is transmitted to progeny cells. Retrospectively, it is possible to determine when the precursors for specific cell types were dividing.

Image collection and cell counting

Images of labeled gonads were collected using a Zeiss LSM 410 confocal microscope. To determine a representative number of cells proliferating per gonad, images of the three most interior sections of
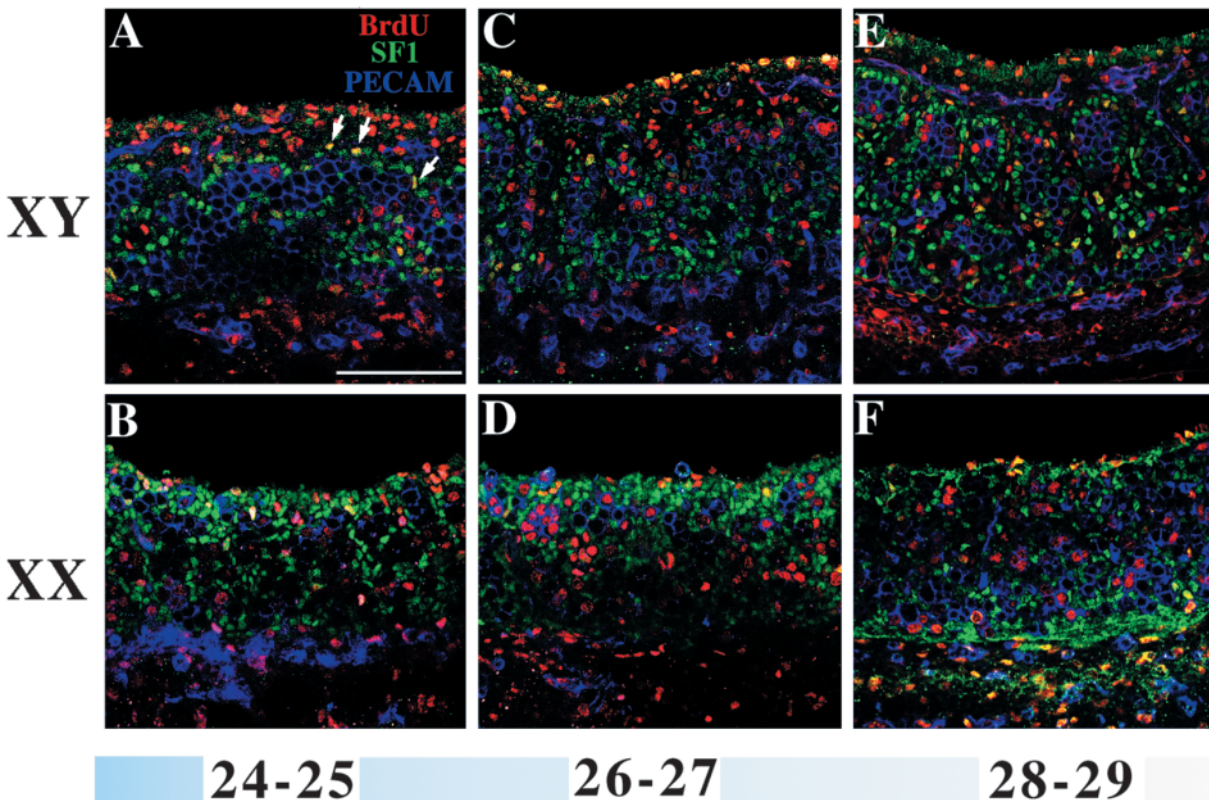
Fig. 2. Confocal images comparing cell proliferation in XY and XX gonads between 24 and 29 ts. The number of dividing somatic cells (red and yellow) continued to increase in the XY gonad during these stages (A,C,E), but remained relatively low in the XX gonad (B,D,F). At 24-25 ts, proliferation in the XY gonad is still concentrated in SF1-negative cells (red) at and near the coelomic epithelium (A). However, by 26-27 ts, many of the proliferating cells at the coelomic epithelium were once again SF1 positive (yellow, C). Rudimentary testis cords are first visible in the XY gonad at 24-25 ts as long clusters of germ cells (blue), adjacent to an arc of SF1-positive pre-Sertoli cells (green). Arrows in A indicate examples of SF1-positive Sertoli cells dividing in the rudimentary cords. The shading of the blue bar approximates the percentage of maximum *Sry* expression present at each stage in XY gonads using RNAse protection. Scale bar, 100 μm.

each gonad were collected using the confocal microscope with a ×40 objective. The plane of section was <2 μm thick using this lens. To avoid counting the same cell in consecutive sections, sections were spaced at 10 μm intervals. The resulting sections were 320 μm^2, roughly the middle third of the gonad at each plane of section. The number of proliferating somatic cells and germ cells were counted in three sections and averaged for each gonad. This number was determined for 8-10 gonads of the same genotype from stages within a range of one or two tail somites, averaged.

RESULTS

An increase in proliferation in the XY coelomic epithelium is the earliest identified hallmark of testis determination

The gonad forms on the ventro-medial surface of the mesonephros and is visible as a distinct ridge of cells by 10.5 dpc in the mouse (reviewed by Capel and Lovell-Badge, 1993). Using RNAse protection, *Sry* expression is first detected in the male genital ridge beginning at 10.5 dpc (8 ts) and ending just after 12.5 dpc (30 ts) (Hacker et al., 1995), by which time the size difference between male and female gonads is already apparent. We examined cellular proliferation in developing gonads using BrdU labeling over the course of *Sry* expression, between 8 and 30 ts.

In the outbred strain CD1, proliferation was identical in the gonads of both sexes between 8 and 16 ts (Figs 1A,B, 3, and data not shown). Proliferation at these early stages was low and often observed in the germ cell population (arrowheads in Fig. 1A,B) and in a somatic cell population at or near the coelomic epithelium (arrows in Fig. 1A,B). SF1, a transcription factor necessary for normal gonad development and the expression of steroidogenic genes (Luo et al., 1995), was detected in most somatic cells at this stage, including the proliferating population at the coelomic epithelium. At this stage, male and female gonads are identical: with the exception of *Sry* expression, no morphological or transcriptional differences have been documented.

The first stage of male-specific proliferation occurred between 16 and 18 ts, just before the peak of *Sry* expression at 18 ts (Hacker et al., 1995). During this stage, somatic cell proliferation nearly doubled in XY gonads but remained constant in XX gonads (Figs 1C,D, 3A). This male-specific proliferation increase was concentrated in SF1-positive cells at or near the coelomic epithelium (Fig. 1C, yellow cells at the coelomic surface).

A second stage of male-specific proliferation was observed between roughly 19 and 25 ts. During this stage, proliferation in the XY gonad continued to increase over that observed in the XX gonad (Fig. 3A). This male-specific proliferation was observed at or directly beneath the XY coelomic epithelium. However, SF1 was no longer strongly detected in this region and proliferation was concentrated in SF1-negative cells (Figs 1E, 2A, red cells at and beneath the coelomic surface). The decrease in the detection of SF1 at the coelomic epithelium was not observed in XX gonads (Figs 1F, 2B). The formation of rudimentary testis cords was first observed late in this stage, consisting of clusters of germ cells surrounded by a layer of Sertoli cells (Fig. 2A).

After 25 ts, proliferation continued to increase in the XY gonad. However, proliferating somatic cells were no longer concentrated at the coelomic epithelium; instead, a mixture of SF1-positive and -negative proliferating cells were observed throughout the XY gonad. Somatic cell proliferation in the XX gonad remained largely in SF1-positive cells at or near the coelomic epithelium (Figs 1, 2), and was relatively constant over the period observed (8-29 ts), with only a slight increase observed after 22 ts (Fig. 3A). Germ cell proliferation showed no significant differences between the sexes during the period observed (8-29 ts) and gradually increased (Fig. 3B).

B6 mice carrying a Y^{POS} allele of *Sry* do not undergo male-specific proliferation

B6 is an inbred mouse strain that undergoes normal sex determination. However, when mice from this strain carry a Y chromosome from *Mus domesticus poschiavinus* mice (Y^{POS}), testis determination fails. These B6 XY^{POS} mice develop either ovaries or ovotestes (a gonad containing ovarian and testicular tissue) (Eicher et al., 1982). This failure of testis formation in B6 XY^{POS} mice is presumably due to the allele of *Sry* on the Y^{POS} chromosome, as this defect can be corrected with an *Sry* transgene from a *Mus musculus* strain (Eicher et al., 1995). However, the presence of the Y^{POS} chromosome in *Mus domesticus poschiavinus* mice, and in most outcrosses of B6 to other inbred strains, leads to normal testis formation. This suggests that the B6 strain carries autosomal variants of genes responsible for the failure in testis development when the *Sry* allele on the Y^{POS} chromosome is present (Eicher et al., 1996). We observed some strain differences between the proliferation patterns of the inbred B6 and the random bred CD1 strains. The 'basal' level of proliferation (the number of proliferating cells observed in the female and in the male before 17 ts) was somewhat higher in B6 than in CD1 gonads during this period. In addition, the SF1-negative stage of proliferation was observed for only 2-3 ts, compared to a period of approx. 5 ts in CD1 gonads (data not shown).

To determine whether the disturbance in testis formation in B6 XY^{POS} mice is coincident with disturbances in the proliferation pattern normally observed in the XY gonad, we compared proliferation in gonads from normal B6 XY and B6 XX mice with gonads from B6 XY^{POS} mice at early stages of sex determination (13-29 ts). Before 20 ts, proliferation was somewhat variable in B6 XY^{POS} gonads, but the level of proliferation was similar to or somewhat lower than in B6 XX littermates (Fig. 4A,C and data not shown). By 20 ts, proliferation in B6 XY^{POS} gonads was consistent with proliferation in B6 XX female littermates and well below that observed in normal B6 XY gonads (Fig. 4E, and data not shown). B6 XY^{POS} gonads examined through 29 ts showed no evidence of a male level of proliferation in either SF1-positive or -negative cells. These gonads also showed no sign of incipient cord formation and were much smaller than normal B6 XY gonads. These results indicated that the presence of the Y^{POS} chromosome disrupted both SF1-positive and -negative stages of male-specific proliferation and these effects correlated with a decrease in the size of the XY^{POS} gonad and a failure to undergo cord formation at the appropriate stage of development.

Sry is the only gene on the Y chromosome required to induce male-specific proliferation in the gonad

B6 XX mice carrying a multicopy transgene of *Sry*

(*TgN(Sry)4Ei*, abbreviated as B6 XX/*Sry*) are completely sex reversed and develop testes (Eicher et al., 1995). Proliferation in the gonads of B6 XX/*Sry* mice was compared to proliferation in gonads of normal B6 XY and B6 XX mice. After 16 ts, proliferation increased in both B6 XX/*Sry* and B6 XY gonads over that observed in B6 XX gonads (Fig. 4 and data not shown). As in CD1 XY gonads, the proliferation increase was observed in both SF1-positive and SF1-negative stages (Fig. 4D,F). B6 XX/*Sry* gonads also showed a male-specific size increase and formed cords with a timing indistinguishable from both CD1 and B6 XY gonads. These experiments demonstrate a tight association between male-specific proliferation and cord formation and prove that *Sry* is the only gene from the Y chromosome required to induce the male-specific increase in proliferation in the gonad.

Proliferation in the SF1-positive and SF1-negative stages gives rise to different lineages within the testis

Two stages of proliferation were identified at and near the coelomic epithelium of gonads that form testes (CD1 XY, B6 XY and B6 XX/*Sry*). During the first stage (8-18 ts in CD1 XY mice), proliferation was concentrated in SF1-positive cells. During the second stage (19-25 ts in CD1 XY mice), proliferation was concentrated in SF1-negative cells. After cord formation, SF1 is localized to Leydig and Sertoli cells (Fig. 2E and Hatano et al., 1994). At earlier stages, SF1 is thought to identify the precursors of these cells. Therefore, proliferation of SF1-positive cells during the first stage may give rise to different lineages than proliferation of SF1-negative cells during the second stage.

To determine the fate of proliferating cells, we injected BrdU into pregnant females at stages of fetal development between 8 and 25 ts. After one hour, this pulse was chased with excess thymidine, and gestation was allowed to continue. In gonads dissected three hours after the thymidine chase, many labeled cells were found beneath the coelomic epithelium during both the SF1-positive and SF1-negative stages of coelomic epithelial proliferation, implying that proliferating cells move into the gonad during both stages (Fig. 5A,D). This finding is consistent with cell lineage tracing experiments using DiI to label coelomic epithelial cells in cultured gonads. In these experiments, cells labeled at the coelomic epithelium moved into the gonad between 15 and 30 ts (Karl and Capel, 1998).

Due to the lack of specific cell markers and morphological features, it is not possible to identify cell types in the gonad before cord formation. To investigate the fate of cells dividing before cord formation, we allowed fetuses to develop in vivo for several days after the thymidine chase, until cord formation had occurred. In fetuses dissected at 12.8 dpc (approximately 33 ts), the basal lamina surrounding the cords was identified with a laminin antibody (arrowheads in Fig. 5B,E). Inside this barrier, SF1 (a marker for Sertoli cells soon after cord formation, Hatano et al., 1994) labels Sertoli cells enclosing clusters of germ cells (labeled with PECAM, Fig. 5C,F).

When embryos were pulsed with BrdU before 18 ts, intensely labeled cells were observed on the inside margin of the basal lamina (s, arrows in Fig. 5B) in a position characteristic of Sertoli cells. Many of these intensely labeled cells were positive for SF1, not PECAM (which labels germ

cells), indicating that they are Sertoli cells (s, arrows in Fig. 5C). When BrdU was injected between 19 and 23 ts, no intensely labeled cells were observed on the inside surface of the basal lamina (Fig. 5E) and no SF1-positive cells were labeled within the cords (s, arrows in Fig. 5F). This result indicates that Sertoli cell precursors proliferate before 18 ts, during the SF1-positive stage of proliferation in the coelomic epithelium, but do not proliferate during most of the SF1-negative stage. This result parallels DiI labeling experiments that demonstrate a time dependence for the fate of coelomic epithelial cells: Sertoli cells arise from the proliferating coelomic epithelium before 18 ts (in the SF1-positive stage), but do not originate from this area after this stage (during the SF1-negative stage) (Karl and Capel, 1998).

At the end of the SF1-negative stage (roughly 24-25 ts) and during the stages following it, a small number of Sertoli cells were labeled in the pulse/chase experiments (roughly two or three per gonadal section, data not shown). At these stages, rudimentary cords had formed and observations of gonads between 24 and 29 ts that were dissected immediately after BrdU injection indicated a low level of proliferation in Sertoli cells already situated within the cords (Fig. 2A arrows,C,E). Two days after this stage (14.5 dpc), Sertoli cells (located within the cords) are one of the most proliferative populations in the gonad, although this proliferation decreases after birth (Russell et al., 1995; Vergouwen et al., 1991). We conclude that Sertoli cells divide before 18 and after 23 ts, but cease division between these stages, at a time when they initiate cord formation.

As expected, BrdU pulse/chase experiments demonstrated that germ cells proliferated between 8 and 30 ts (Fig. 5C,F). In addition, precursors to both SF1-positive and -negative interstitial cells proliferated during these stages (Fig. 5C,F). The SF1-positive interstitial cells labeled with BrdU during both stages were likely precursors of Leydig cells. We identified many of the BrdU labeled, SF1-negative interstitial cells as vascular cells using the endothelial marker PECAM, indicating that cells that contribute to the vasculature of the testis divide during both stages. However, many cells dividing at these stages were not labeled with any of the available cell specific markers (SF1, Müllerian Inhibiting Substance (MIS), Steroidogenic Acute Regulatory Protein, Wilms' Tumor, Stage Specific Embryonic Antigen 1, or PECAM; Fig. 5 and data not shown).

DISCUSSION

An important goal in the study of mammalian sex determination is the identification of molecular and cellular pathways downstream of *Sry* that control testis development. However, to date no targets of this putative transcription factor have been identified, making identification of direct molecular pathways difficult. We reasoned that a reverse approach would be informative: the investigation of *Sry*-dependent changes in gonad morphology that mark the initiation of testis organogenesis would allow the deduction of molecular and cellular pathways between *Sry* and these changes. In this study, we investigated the etiology of the rapid size increase observed in the rudimentary testis. Our findings attribute the majority of this size increase to an increase in proliferation induced by *Sry*.
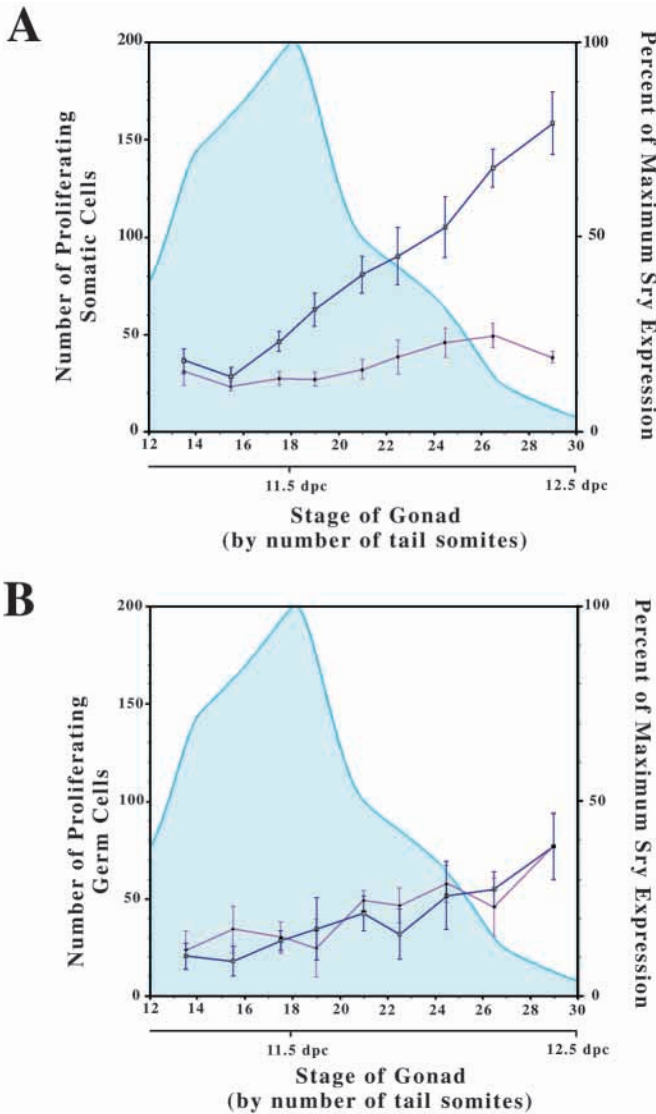
Fig. 3. The average number of somatic (A) and germ cells (B) proliferating per section in XY (dark blue) and XX (pink) gonads at stages between 13.5 and 29 ts were graphed and superimposed on the percentage of maximum *Sry* expression present at each stage in XY gonads (light blue; modeled after Hacker et al., 1995). The corresponding dpc are indicated. Error bars indicate standard deviation. (A) Somatic cell proliferation in the XY gonad increased over that observed in the XX gonad between 15.5 and 17.5 ts, just before the peak of *Sry* expression. Somatic cell proliferation in the XY gonad continued to increase over the experimental time course. Somatic cell proliferation in the XX gonad remained relatively constant, with a slight increase approaching 27 ts. (B) Germ cell proliferation increased gradually over time, but no significant sex-specific differences were observed.

An increase in proliferation is the earliest known marker of testis determination to appear after the initiation of *Sry* expression

During the early stages of gonad formation, somatic cell proliferation was similar in both sexes and largely concentrated at or near the coelomic epithelium, supporting earlier theories that this region is an important contributor of somatic cells to the gonads of both sexes (Whitehead, 1904; Brambell, 1927; Torrey, 1945; Pelliniemi, 1976; Merchant-Larios, 1979). Germ cells were a proliferative population at all stages observed, 10.5-12.5 dpc, confirming a previous study (Tam and Snow, 1981). We detected no significant differences between the proliferation of male and female germ cells, consistent with results indicating that germ cells do not undergo sex-specific differences in proliferation until after 13.5 dpc (McLaren, 1981).

A difference between male and female proliferation was first observed in somatic cells of the gonad between 16 and 18 ts, just before the peak of *Sry* expression. The timing of this proliferation increase confirms a prediction made by Palmer and Burgoyne (1991b), who extrapolated the size difference between male and female gonads in B6 outcrosses at 12.5 dpc and predicted that an increase in the growth rate of the XY

Fig. 4. Confocal images of cell proliferation in B6 XY[POS] and B6 XX/*Sry* gonads between 15 and 21 ts. SF1 labeled a population of somatic cells in the gonad (green). PECAM labeled germ cells and vasculature (blue). The number of dividing cells (red and yellow) in B6 XY[POS] gonads (A,C,E) did not increase beyond the level of normal B6 XX gonads. In B6 XX/*Sry* gonads, an increase in proliferation was observed in both a SF1-positive (yellow cells in D) and SF1-negative (red cells in F) stage at the coelomic epithelium. Scale bar, 100 μm.
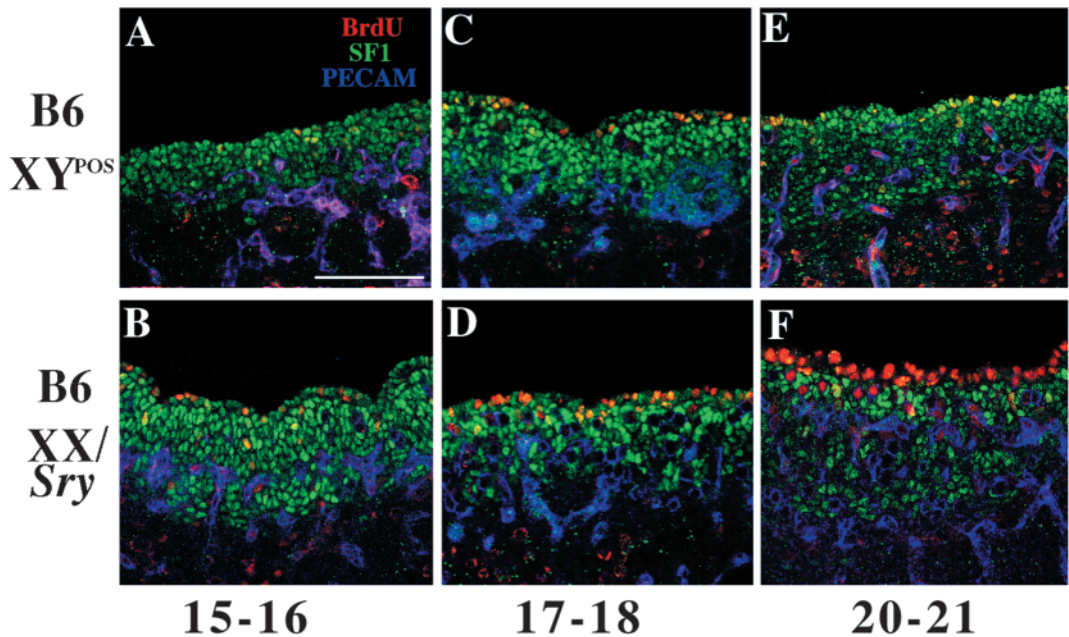
Fig. 5. Confocal images comparing BrdU pulse/chase gonads labeled at different stages of gonad development. Pregnant mice were pulsed with BrdU for one hour before they were injected with excess thymidine, which acts as a chase. After a 3 hour chase, many BrdU-labeled cells (red) were found beneath the coelomic epithelium in gonads pulsed both before (A) and after (D) the 18 ts stage, suggesting that proliferating cells at the coelomic epithelium migrate into the gonad at both stages. By the 33 ts stage, testis cords (tc) had formed in the XY gonad and could be identified using anti-laminin (green in B,E) to label the basal lamina (bl; arrowheads). Only two cell types are found within cords, Sertoli cells (labeled with anti-SF1; green in C,F) and germ cells (labeled with anti-PECAM; blue in C,F). In gonads labeled before 18 ts, cells with a strong BrdU label (s and arrows in B) were found on the inside margin of the basal lamina. These cells double labeled with SF1 (yellow, C), indicating they are Sertoli cells. (The basal lamina is occasionally weakly visible due to background labeling by secondary antibodies: arrowheads, bl in C). In gonads labeled between 19 and 23 ts, BrdU-labeled cells were not found on the inside surface of the basal lamina (E) or among the SF1-positive (green) population within the cords (s, arrows in F), indicating that Sertoli cells do not proliferate at this stage. SF1-positive and -negative interstitial cells were proliferating at both stages (i). The shading of the blue bar approximates the percentage of maximum *Sry* expression detected at each stage (modeled after Hacker et al., 1995). Pulse/chase experiments were initiated at discrete stages throughout the developmental period indicated by the red striped areas. Scale bar, 25 μm.
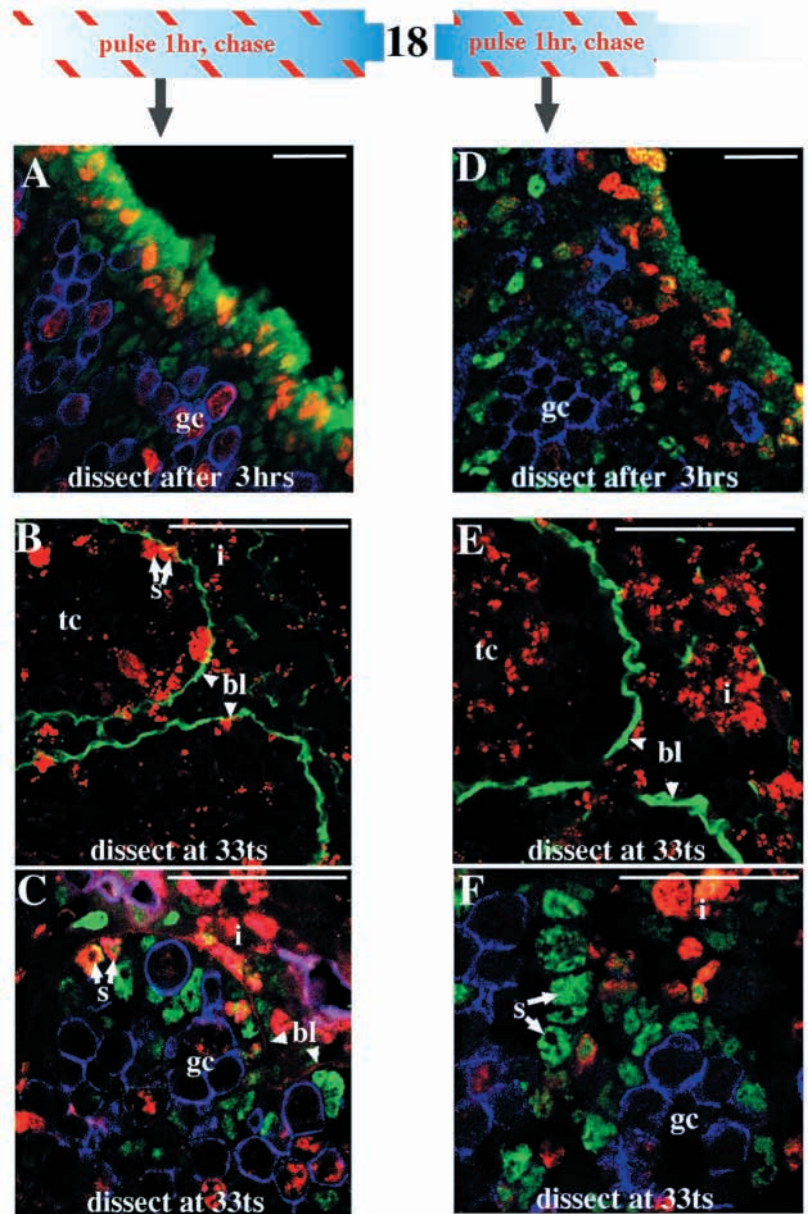
gonad would be first visible at 11 dpc and 6 hours (15.5 ts). Our results indicate that the number of proliferating cells in the coelomic epithelium of XY gonads nearly doubled compared to the number observed in XX gonads at a time prior to the appearance of any other known markers of testis development, such as MIS expression (Hacker et al., 1995) or cord formation. Therefore, this increase in proliferation at the XY coelomic epithelium is the earliest documented difference between XY and XX gonads to occur after the initiation of *Sry* expression.

The male-specific increase in proliferation is correlated with normal testis development and is dependent on *Sry*

The partial or complete failure of testis formation in B6 XY^{POS} mice (Eicher et al., 1982) was coincident with a failure to initiate the male-specific proliferation increase, despite the presence of a Y chromosome and a single dosage of the X chromosome. The reduction in cell number that is observed in XY^{POS} gonads could lead to a decrease in the total amount of *Sry* expressed and a subsequent decrease in the levels of signals downstream of *Sry* that normally induce male-specific effects. For example, recent results indicate that male-specific migration into XY^{POS} gonads is also impaired (Albrecht, Capel, Washburn and Eicher, personal communication).

The formation of testes in B6 XX mice carrying transgenic copies of *Sry* was coincident with the appearance of both stages of the proliferation increase normally observed in B6 XY gonads, despite the absence of other genes on the Y chromosome. This evidence demonstrated that *Sry* is the only gene on the Y chromosome required to induce both stages of male-specific proliferation. We also concluded that the presence or absence of male-specific proliferation is tightly correlated with the presence or absence of normal testis development.

Proliferation of Sertoli cells may be a cell autonomous effect of *Sry*

Cells in the XY coelomic epithelium appear to act as a stem cell population, where continuous divisions give rise to many of the somatic cells in the testis. Proliferation in this area occurred in at least two stages, with the precursors of different cell types dividing during different stages. BrdU pulse/chase labeling indicated that Sertoli cell precursors divided during the first stage, at a time when proliferation in the gonad was concentrated in SF1-positive cells in the coelomic epithelium.

DiI lineage tracing experiments also indicate that Sertoli cells originate from the coelomic epithelium during the first stage (prior to 18 ts) (Karl and Capel, 1998). After the first stage of proliferation, Sertoli cells ceased dividing temporarily (18-23 ts). This quiescent period occurred just before the first testis cords became visible, at a time when Sertoli cells move into the interior of the gonad (Karl and Capel, 1998), begin to aggregate (Magre and Jost, 1991) and initiate cord formation. Sertoli cells resume division after 23-24 ts, suggesting that the arrest of cell division has a function in the migration and differentiation of Sertoli cells prior to the formation of testis cords.

There are several lines of evidence that indicate that the precursors of Sertoli cells express *Sry*. (1) Sertoli cells are the first cells of the testis to differentiate (Magre and Jost, 1991). (2) Sertoli cells are the only cells in the testis that show a bias for the presence of a Y chromosome (and presumably *Sry*) during the determination of the testis in XY↔XX chimeric mice (Palmer and Burgoyne, 1991b). (3) Directing expression of an *Sry* transgene to the Sertoli cell lineage sex reverses XX mice (Swain et al., 1998). Because Sertoli precursors proliferate at the time and place where the first male-specific increase is observed and because Sertoli precursors are thought to express *Sry* during this period, we propose that the early *Sry*-dependent increase in the proliferation of SF1-positive cells at the coelomic epithelium is the result of a cell autonomous pathway initiated by *Sry* in Sertoli cell precursors. This proposed cell autonomous action of *Sry* is also supported by the observation that the proliferation increase is the earliest known effect of *Sry*.

A second stage of *Sry* dependent proliferation occurs in a variety of lineages

The Sertoli cell progeny of the early divisions in the SF1-positive coelomic epithelium move into the gonad and leave behind a population of proliferating SF1-negative cells that do not give rise to Sertoli cells. This second stage of proliferation also is dependent on *Sry*. BrdU pulse/chase experiments indicated that the precursors of vascular and pre-Leydig cells divide during this stage. XY↔XX chimeras indicated that these lineages can be derived from either XX or XY cells (Palmer and Burgoyne, 1991a), indicating that differentiation and proliferation of these cells is probably not a cell autonomous effect of *Sry*. Many of the cells derived from proliferation during the SF1-negative stage were not labeled with any of the available markers for testis cells. It is possible that these cells are not fully differentiated, or that they belong to an unknown lineage. Many unidentified cells remained at the coelomic epithelium and probably contributed to the tunica's cellular layers of fibroblasts and myocytes.

Cell proliferation has multiple roles in organ formation

Cell division is a critical component of organ formation. This process is important not only in generating the number of cells needed in a multicellular organ, but it is also closely integrated with many other developmental processes. For example, cell diversity can be generated through asymmetric division (Horvitz and Herskowitz, 1992) or by rapid proliferation which moves cells away from the influence of a signaling center (Tabin, 1998). Some biological clocks depend on biochemical

oscillations during cycles of cell division (reviewed by Pourquie, 1998). It has also been postulated that proliferation alters the transcriptional accessibility of the *Hox* gene clusters, thereby controlling the timing of the expression of specific genes and consequent cell differentiation (Duboule, 1994; Ohsugi et al., 1997).

We have shown that *Sry* induces a rapid increase in proliferation in the mouse XY gonad. This male-specific proliferation is initially observed in Sertoli cell precursors and is one of the forces behind the size increase of the male gonad observed at the outset of testis organogenesis. An increase in the size of the testis compared to the ovary has been documented at the earliest stages of sex determination in many mammals, such as mice, rats, sheep and humans. This early size difference also is observed in species that are not known to possess *Sry*, or even a Y chromosome, such as chickens, turtles and alligators (reviewed by Mittwoch, 1986). In alligators, proliferation in Sertoli cell precursors is thought to be the first sign of male sex determination (Smith and Joss, 1993), indicating that proliferation of these critical cells may be a common element in the testis pathway. Based on such commonalities among species that use disparate mechanisms of sex determination it has been proposed previously that differential growth of the testis determines the sex of the organism (Mittwoch, 1986). Our data shows that an increase in proliferation is a consequence of *Sry* function and may be an important mechanism involved in the initiation of testis organogenesis in vertebrates.

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