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Evidence That *Sry* Is Expressed in Pre-Sertoli Cells and Sertoli and Granulosa Cells Have a Common Precursor

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The expression of *Sry* in the undifferentiated, bipotential genital ridges of mammalian XY fetuses initiates testis development and is hypothesized to do so by directing supporting cell precursors to develop as Sertoli cells and not as granulosa cells. To directly test this hypothesis, transgenic mice expressing EGFP under the control of the *Sry* promoter were produced. After establishing that the transgene was expressed in fetal gonads similarly to endogenous *Sry*, the spatial and temporal expression of the *Sry*-EGFP transgene was investigated in developing gonads by using confocal microscopy and immunofluorescent histochemistry. This analysis indicated: (1) *Sry* is first expressed in cells located centrally in the genital ridge and then later in cells located at the cranial and caudal poles, (2) *Sry* is expressed exclusively in pre-Sertoli cells in the urogenital ridge, and (3) Sertoli and granulosa cells develop from a common precursor. These results support the hypothesis that *Sry* initiates testis differentiation by directing the development of supporting cell precursors as Sertoli rather than granulosa cells. Furthermore, the *Sry* expression pattern explains the nonrandom distribution of testicular and ovarian tissue in mammalian ovotestes.

***Key Words: Sry;* EGFP; Sertoli cell; granulosa cell; sex determination; gonadogenesis; organogenesis.**

INTRODUCTION

Mammalian testes and ovaries share a common precursor: the undifferentiated, bipotential genital ridge. To differentiate into an ovary or a testis, the genital ridge must contain complete sets of female and male cell lineage precursors, or each lineage precursor must be capable of adopting an ovarian or testicular cell-type fate. The "complete set" hypothesis was proposed by Witschi, who suggested that the ovary develops from the gonadal cortex (coelomic epithelium) whereas the testis develops from the medulla (Witschi, 1951). The "common precursor" hypothesis (Gillman, 1948) is currently in favor and is supported by data from cell lineage-tracing experiments (Karl and Capel, 1998; Capel, 2000).

The common precursor hypothesis is best viewed as a series of bipotential cell fate decisions within the four cell lineages that comprise the gonad: the germ cells, connective tissue cells, steroid producing cells, and supporting cells. The primordial germ cells originate outside the gonad and then migrate to and populate the genital ridge (reviewed in McLaren, 2000).

The remaining three cell lineages are somatic and recent experiments have greatly increased our knowledge of their developmental origin. Experimental data from the mouse suggest that at least some testicular connective tissue cells (peritubular myoid, vascular endothelial, and myoepithelial cells) are derived from mesenchymal cells that migrate into the gonad from the adjacent mesonephros (Buehr *et al.*, 1993; Martineau *et al.*, 1997). This male-specific migration is intriguing because peritubular myoid cells are required for testis cord development and appear to have no ovarian homologue. Whether mesonephros-derived cells are the origin of connective tissue cells in the ovary is unclear, but, if they are, these cells must migrate into the developing mouse ovary before E11.5 (E, embryonic day) or after E16.5 (Martineau *et al.*, 1997).

Additional data, again from the mouse, suggest that the steroid-producing cells (Leydig cells in the testis and theca cells in the ovary) originate from cells that migrate into the genital ridge from the mesonephros in a nonsex-specific manner before E11.5 (Hatano *et al.*, 1996; Merchant-Larios and Moreno-Mendoza, 1998; Capel, 2000).

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The final cell lineage is the supporting cells (Sertoli cells in the testis and granulosa cells in the ovary). Sertoli cells originate from the coelomic epithelium covering the genital ridge and migrate from this site into the developing testis (Karl and Capel, 1998). Coelomic epithelium cells also migrate into the developing ovary, but their fate is unknown because appropriate markers are not yet available. However, if Sertoli and granulosa cells share a common precursor, then the coelomic epithelium is the origin of granulosa cells.

Sry (sex determining *r*egion *Y* chromosome) is the Y chromosome gene responsible for initiating testis development (Sinclair *et al.*, 1990; Koopman *et al.*, 1991). The current model for mammalian sex determination suggests that expression of *Sry* in the genital ridge initiates testis development by directing supporting cell precursors to develop as Sertoli rather than granulosa cells (Burgoyne *et al.*, 1988a) and that the development of all other gonadal cell types is dependent on Sertoli cell differentiation (Magre and Jost, 1980). This model is based on the facts that the first somatic cells to differentiate in the developing testis are Sertoli cells (Magre and Jost, 1980) and Sertoli cells are predominately XY in gonads of opposite sex (XX ↔ XY) chimeric mice (Burgoyne *et al.*, 1988a; Palmer and Burgoyne, 1991; Patek *et al.*, 1991). At present, the expression of *Sry* in pre-Sertoli cells has not been demonstrated directly, partly because no antibody to mouse SRY is available and because RNA *in situ* hybridization experiments generally give poor morphological detail.

The above model for mammalian sex determination requires two hypotheses to be true: (1) *Sry* is expressed in Sertoli cells, and (2) Sertoli and granulosa cells share a common precursor. We report here results from experiments designed to directly test these two hypotheses. To accomplish this, mice carrying a transgene in which the *Sry* coding region was replaced with the *e*nhanced *g*reen *f*luorescent *p*rotein (EGFP) gene were produced. After first establishing that the transgene was expressed in fetal gonads at the time when endogenous *Sry* is expressed, the spatial and temporal expression of EGFP in developing gonads was explored by using confocal microscopy and immunofluorescence histochemistry. The results presented here indicate that both hypotheses are correct: *Sry* is expressed in pre-Sertoli cells and Sertoli and granulosa cells have a common precursor. In addition, the results indicate that *Sry* is first expressed in cells located centrally in the genital ridge and then later in cells located at the cranial and caudal poles. Based on this expression pattern, we present a hypothesis to explain the nonrandom distribution of testicular and ovarian tissue in mammalian ovotestes.

MATERIALS AND METHODS

Sry-EGFP Transgenic Mice

The *Sry* promoter region was subcloned from p741 [a plasmid subclone of L741 (Gubbay *et al.*, 1990)] into the pEGFP-1 promoter reporter vector (Clontech) as follows (Fig. 1). A fragment of the *Sry* promoter/5′ UTR was PCR amplified by using primers Sry-8024 (5′-GTTACAAGCACATTTTGGTCAGTGG) and Sry-8317 (5′-ACATGGCCCTCGAGGCTCTCTAG). (All numbering refers to GenBank entry X67204 for clone L741.) Primer Sry-8317 was designed so that the ATG start codon at position 8304 was changed to GAG (underlined above) to create an *Xho*I restriction site. The PCR was digested with *Eco*RV and *Xho*I, gel purified, and inserted into pBluescript-SK+ (Stratagene) by using standard methods. The nucleotide sequence of the PCR fragment in this clone, pBS-Sry(*Eco*RV–*Xho*I), was verified by sequencing. A *Pst*I–*Eco*RV (bp 542-8244) fragment of p741 was then subcloned into pBS-Sry(*Eco*RV–*Xho*I) creating pBS-Sry(*Pst*I–*Xho*I) that contained the *Sry* upstream region from bp 542-8304. The *Sry* promoter region was then subcloned into pEGFP-1 by digesting pBS-Sry(*Pst*I–*Xho*I) with *Pst*I and *Kpn*I and inserting this fragment into the *Pst*I and *Kpn*I sites in the pEGFP-1 multiple cloning site upstream of the EGFP coding region, thereby creating the pSry-EGFP plasmid.

To produce transgenic mice, pSry-EGFP was digested with *Pst*I and *Sph*I and gel purified. This fragment, containing the *Sry* promoter driving EGFP expression, was microinjected into the pronucleus of C57BL/6 J fertilized eggs by using standard methods (Fig. 1) (Hogan *et al.*, 1994). It should be noted that this construct does not contain the endogenous *Sry* 3′ UTR, but rather an artificial one with two SV40 early mRNA polyadenylation signals. Putative transgenic offspring were screened for the presence of the EGFP gene by PCR using primers EGFP-594 (5′-CAAGATCCGCCACAACATCG) and EGFP-763 (5′-CCAGCAGGACCATGTGATCG) multiplexed with myogenin control primers (5′-TTACGTCCATCGTGGACAGCAT and 5′-TGGGCTGGGTGTTAGTCTTAT). To detect the presence of a Y chromosome, these two primer pairs were multiplexed with primers 5′-CTGGAGCTCTACAGTGATGA and 5′-CAGTTACCAATCAACACATCAC, which detect the Y chromosome locus YMT2/B (Capel *et al.*, 1999).

Seven transgenic founders were recovered and lines were established from each. Transgenic mice from five lines expressed *EGFP* RNA as assessed by RT-PCR analysis of E11.5 gonads (see below). (This is the time when endogenous *Sry* is maximally expressed.) Gonads from E12–E12.5 fetuses from these lines were examined for EGFP protein expression by using confocal microscopy (see below). Both EGFP autofluorescence and GFP antibody staining were examined. One transgenic line, designated C57BL/6-J-TgN(*Sry-EGFP*)92Ei or Tg92, expressed both EGFP RNA and visible protein and was used in the studies reported here. The Tg92 line was maintained by interbreeding transgenic carriers at each generation.

Fetal and Postnatal Gonads

Unless otherwise stated, Tg92 mice used for analysis were homozygous for the transgene and from the F_3 and later generations. (EGFP expression is visible in Tg92 heterozygotes, but is more easily detected in presumptive homozygotes; data not shown). Fetuses were collected from timed matings. For overnight matings, noon on the day a mating plug was observed is designated as E0.5 (embryonic day). The day of birth is designated as P0 (postnatal day). For more precise staging of fetuses younger than E13.0, the number of tail somites posterior to the hind limb bud was counted: E10.5 is ~8 tail somites (ts), E11.5 is ~18ts, and E12.5 is ~28–30ts (Hacker *et al.*, 1995). Fetuses older than E13.0 were staged by forelimb and hindlimb morphology (Theiler, 1989).

Paired urogenital ridges, gonad/mesonephros complexes, or gonads were dissected, all nongonadal and nonmesonephric tissue was carefully removed, and the mesonephros was trimmed to the length of the gonad for RT-PCR analysis. Tissue from each fetus was digested in PCR buffer supplemented with Proteinase K and detergents, and 2 μl was used as template for multiplex PCR genotyping as outlined above.

RT-PCR

RNA was extracted from paired urogenital ridges, gonad/mesonephros complexes, or single gonads by using the RNeasy Mini Kit (Qiagen). Lysed tissue was stored at −80°C in RLT buffer until processed. The RNA was DNased by using either an on-column protocol (Qiagen) or after elution from the column by using the DNA-free protocol (Ambion). Two microliters of each 30-μl RNA sample were tested for DNA contamination by PCR amplification using the EGFP-594 and EGFP-763 primers (40 cycles). Any sample contaminated with DNA was reDNased, purified, and retested.

One-third (10 μl) of the RNA sample was reverse transcribed at 42°C for 1 h in a 20-μl reaction by using the RNA PCR Kit (Applied Biosystems). Parallel reactions were performed, one with reverse transcriptase and one without. A no template (H_2O) negative control was included in each experiment. The reverse transcription reaction (2 μl) was PCR amplified with primers specific for the *Hprt* gene (5′-CCTGCTGGATTACATTAAAGCACTG and 5′-GTCAAGGGCATATCCAACAACAAAC) (Koopman *et al.*, 1989) as a positive control for the presence of intact RNA. The initial screening of transgenic lines for *EGFP* expression was accomplished by using the EGFP-594 and EGFP-763 primer pair and 4 μl of the RT reaction as template in a 50-μl standard PCR. Subsequent expression analyses employed the AmpliTaq Gold hot-start protocol (Applied Biosystems) using 2 μl of the RT reaction in a 25-μl reaction.

Whole-Mount Indirect Fluorescent Immunohistochemistry

Tissue samples were fixed in 4% paraformaldehyde in PBS overnight at 4°C, rinsed twice in PBS, quenched in 50 mM NH_4Cl for 15 min, and blocked for at least 24 h in blocking buffer (1% BSA, 0.1% Saponin, 0.02% sodium azide in PBS).

Samples for whole-mount analysis were then incubated for at least 24 h in primary antibody diluted in fresh blocking buffer, washed five times for 1 h each in blocking buffer, incubated for at least 24 h in secondary antibody diluted in fresh blocking buffer, and washed five times for 1 h each in blocking buffer. A modified protocol was used for E13.5 gonads stained for EGFP, AMH, and PECAM-1 expression because the anti-GFP and anti-AMH antibodies both were raised in rabbit. Samples were incubated in anti-GFP antibody for 24 h, washed five times for 1 h each in blocking buffer, and incubated in Cy5-conjugated Fab fragment goat anti-rabbit IgG for 24 h. Samples then were washed as before, postfixed in 4% paraformaldehyde for 2 h at 4°C, washed in PBS twice for 15 min, and reblocked in blocking buffer overnight. Samples were then incubated in anti-AMH and anti-CD31 primary antibodies, washed as usual, incubated in appropriate secondary antibodies, and washed again as usual. All incubations and washes were carried out at 4°C on an orbital shaker.

Cryostat-sections of P1 and P5 ovaries also were analyzed. Ovaries were prepared and sectioned at 10 μM by using standard protocols. Sections were processed on slides for immunohistochemistry as follows. Sections were quenched in NH_4Cl as above and blocked in blocking buffer supplemented with 10% fetal calf serum (FCS) overnight at 4°C in a humidified chamber. Because the anti-GFP antibody and the anti-LAMININ antibody both were raised in rabbit, an immunohistochemistry protocol similar to that described above was used. Sections were incubated in anti-GFP antibody overnight, washed three times for 30 min in blocking buffer without FCS, and incubated in Cy5-conjugated Fab fragment goat anti-rabbit IgG overnight at 4°C. Samples then were washed as before, postfixed in 4% paraformaldehyde for 2 h at 4°C, washed in PBS twice for 15 min, incubated in anti-LAMININ antibody overnight, washed, incubated in Cy3-conjugated secondary antibody, washed, and couterstained with YOYO-1 iodine. All samples were mounted in SlowFade-Light Antifade (Molecular Probes).

The primary antibodies used and pertinent details are listed in Table 1. NCAM and ITGA6 expression was analyzed but was uninformative in regard to EGFP-expressing cells and is not discussed further. FITC, Cy3-, or Cy5-conjugated secondary antibodies were from Jackson ImmunoResearch and used at 1:500 dilution. The Cy5-conjugated Fab fragment goat anti-rabbit IgG antibody was from Jackson ImmunoResearch and used at 1:100 dilution. The Alexa Fluor-488-conjugated secondary antibodies were from Molecular Probes and used at 1:500 dilution. Nuclei were visualized by counterstaining in 1 mM TO-PRO-3 iodine or 10 μM YOYO-1 iodine (Molecular Probes), which bind to DNA.

The specificity of the anti-GFP antibody was assessed by staining gonads from a strain that contains an X-linked ubiquitously expressed EGFP transgene [TgN(GFPX)4Nagy] (Hadjantonakis *et al.*, 1998). Because this transgene is subject to random X-inactivation, ~50% of the cells in heterozygous XX females and all cells in hemizygous XY males should express EGFP. Indeed, this was observed (data not shown). More importantly, all EGFP autofluorescent cells were positive for anti-GFP antibody staining and EGFP-negative cells were negative for anti-GFP antibody staining. No EGFP autofluorescence or anti-GFP antibody staining was observed in control nontransgenic gonads (data not shown). Both the fluorescent signal intensity and signal/background ratio was greater for the anti-GFP antibody and indirect immunofluorescence than for EGFP autofluorescence (see below).

Image Collection and Processing

Fluorescently labeled samples were imaged by using a Leica TCS-NT laser scanning confocal microscope. Whole-mount samples were viewed in antifade buffer with glycerol in a culture dish with attached coverslip. For all Z-series, the section interval was 2 μm. Confocal microscope images were assembled by using MetaMorph (Universal Imaging) and Photoshop 6 (Adobe).

RESULTS

An Sry-EGFP Transgene as a Reporter for Sry Expression

The *Sry*-EGFP transgene construct was designed to be a promoter reporter without the *Sry* coding region so that expression could be analyzed in developing testes and

ovaries (Fig. 1). Therefore, sex reversal of XX mice could not be used to determine whether the transgene was functioning similar to endogenous *Sry*. Thus, the temporal expression of the transgene was assessed in urogenital ridges (E10.5 and E11.5) and isolated gonads (E15.5, P1, and P28) by using a qualitative RT-PCR assay and compared to the known temporal expression of *Sry* (data not shown). Endogenous *Sry* is detectable in XY urogenital ridges between E10.5 and ~E13.0 using RT-PCR with peak expression at E11.5 (Koopman *et al.,* 1990). Because the amount of RNA present in urogenital ridges is very low, one-third of the RNA extracted from each sample was used in the EGFP RT-PCR assay. Although the RT-PCR assay was not quantitative, it appeared that *EGFP* RNA was present at low levels at E10.5, at higher levels at E11.5, and then again at low levels at E15.5 in XX and XY urogenital ridges. However, at E15.5, the amount of *EGFP* RNA was noticeably greater in ovaries than in testes (especially considering that ovaries are much smaller than testes at this time and therefore less RNA was used in the assay). *EGFP* RNA also was present in XX and XY gonads from P1 and P28 animals, but at very low levels. The RT-PCR results suggest Tg92 is up- and down-regulated similarly to endogenous *Sry*.

The expression of EGFP in samples analyzed by confocal microscopy suggests Tg92 is spatially expressed similarly to endogenous *Sry*. In mice, *Sry* is expressed in the genital ridge and not in the adjacent mesonephros or metanephric kidney (Jeske *et al.,* 1995; Koopman *et al.,* 1990). Many genital ridge/mesonephros complexes were examined in the course of this study and EGFP expression was observed only in the genital ridge/gonad and not the mesonephros (see Figs. 2 and 3 for example). In addition, EGFP expression was not observed in three pairs of metanephric kidneys examined from E12–E12.5 fetuses, including two pairs that were examined by using the anti-GFP antibody (data not shown). We conclude that Tg92 is an accurate reporter of *Sry* expression.

Sry Is Expressed Exclusively in Gonadal Somatic Cells

Previous experiments indicated that *Sry* is expressed exclusively in somatic cells in the male fetal genital ridge (Koopman *et al.,* 1990; Rossi *et al.,* 1993). To determine what cell type expressed Tg92, the expression of a number of marker proteins was examined in whole-mount urogenital ridges by using three-color confocal microscopy and indirect fluorescent immunohistochemistry. Initially, the proteins examined included two somatic cell markers and one germ cell marker (Table 1). As expected, GATA4 was expressed in the nucleus of gonadal somatic cells but not in germ cells or mesonephric cells (Viger *et al.,* 1998) (Figs. 2B and 4B). WT1 was expressed in the nucleus of gonadal somatic cells and mesonephric cells but not in germ cells (Pelletier *et al.,* 1991; Armstrong *et al.,* 1992) (Figs. 4E and 4F). Conversely, PECAM-1 was expressed on the cell surface of primordial germ cells and vascular endothelial cells in the gonad and mesonephros (Martineau *et al.,* 1997) (Figs. 2C, 2D, and 2F). We found that PECAM-1-positive vascular endothelial cells did not express GATA4 or WT1 (Figs. 2D, 2F, and 4D). This finding is not unexpected because these cells migrate into the genital ridge from the mesonephros.

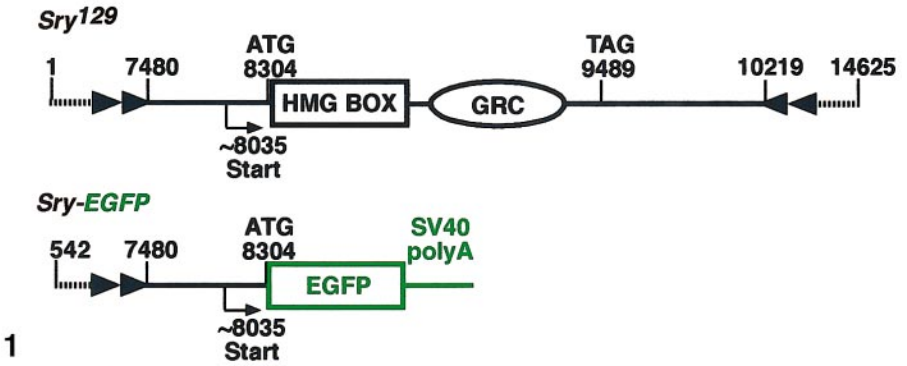
In XX and XY genital ridges isolated from Tg92 fetuses, all cells that expressed EGFP also expressed GATA4 and WT1 but not PECAM-1 (Figs. 2D and 2F). However, only a subset of GATA4- and WT1-expressing cells also expressed EGFP. Furthermore, at all developmental stages examined, EGFP-producing cells expressed only somatic cell markers and never germ cell markers. We conclude that *Sry* is expressed in a subset of gonadal somatic cells and not in germ cells or vascular endothelial cells.

Sry-EGFP Expression in Undifferentiated Genital Ridges: E11–E12 (12–23ts)

Next, the temporal and spatial expression of EGFP in undifferentiated urogenital ridges was examined. XX and XY gonads appear essentially identical at the light microscope level before the initiation of testis cord development in XY gonads at ~E12. A similar result was observed for EGFP-expressing cells (Figs. 2D and 2F). EGFP-positive cells had a mesenchymal morphology in both XX and XY gonads. Furthermore, the temporal and spatial expression of EGFP also was essentially identical. EGFP was first visible only when the signal was amplified by using the anti-GFP antibody in a few cells deep in the center of genital ridges from fetuses at ~E11 (13ts) (Fig. 3A). (EGFP expression was not detectable in four urogenital ridges from XX fetuses at the 12ts stage.) At progressively later stages, EGFP autofluorescence became visible, the number of EGFP-expressing cells in the central region of the gonad increased, and EGFP-positive cells also were present nearer to the gonad surface and cranial and caudal poles (Figs. 3B–3D). By E11.5 (17–20ts), EGFP-positive cells were present in the middle 70–80% of the genital ridge. Independently, a similar center-to-poles expression pattern was detected by RNA *in situ* hybridization (Bullejos and Koopman, 2001).

EGFP-expressing cells were never detected in the coelomic epithelium, from which Sertoli cells originate (Figs. 3E and 3F). (This is also consistent with the above *Sry in situ* hybridization results.) However, at the earliest stages, EGFP-positive cells were present in the region immediately below it and more EGFP-expressing cells were observed nearer to the coelomic epithelium than to the mesonephros. Once EGFP-positive cells began to occupy a significant portion of the genital ridge, the number of EGFP-positive cells immediately below the coelomic epithelium decreased.

Generally, more EGFP-expressing cells were present toward the caudal than the cranial pole (Fig. 3D). For example, the distribution of EGFP-positive cells was analyzed in 26 gonads from fetuses between 13 and 22ts: 12 had EGFP-



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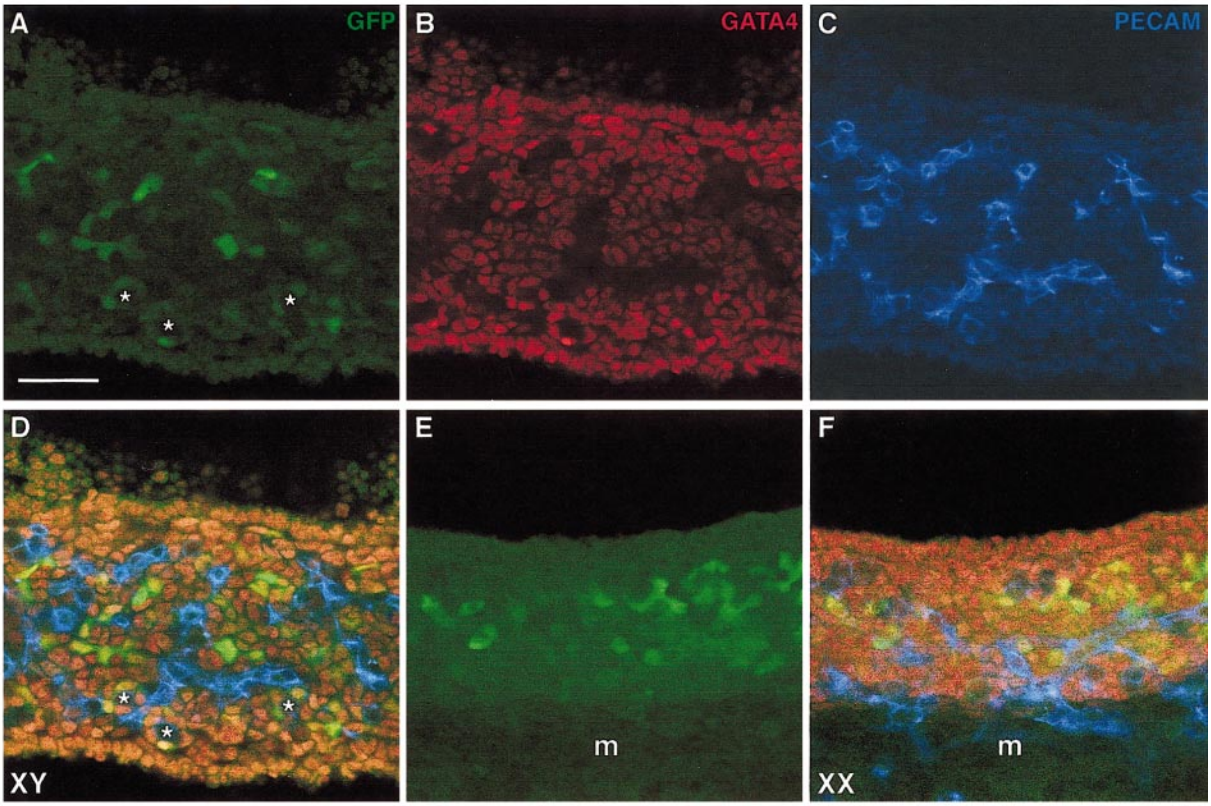

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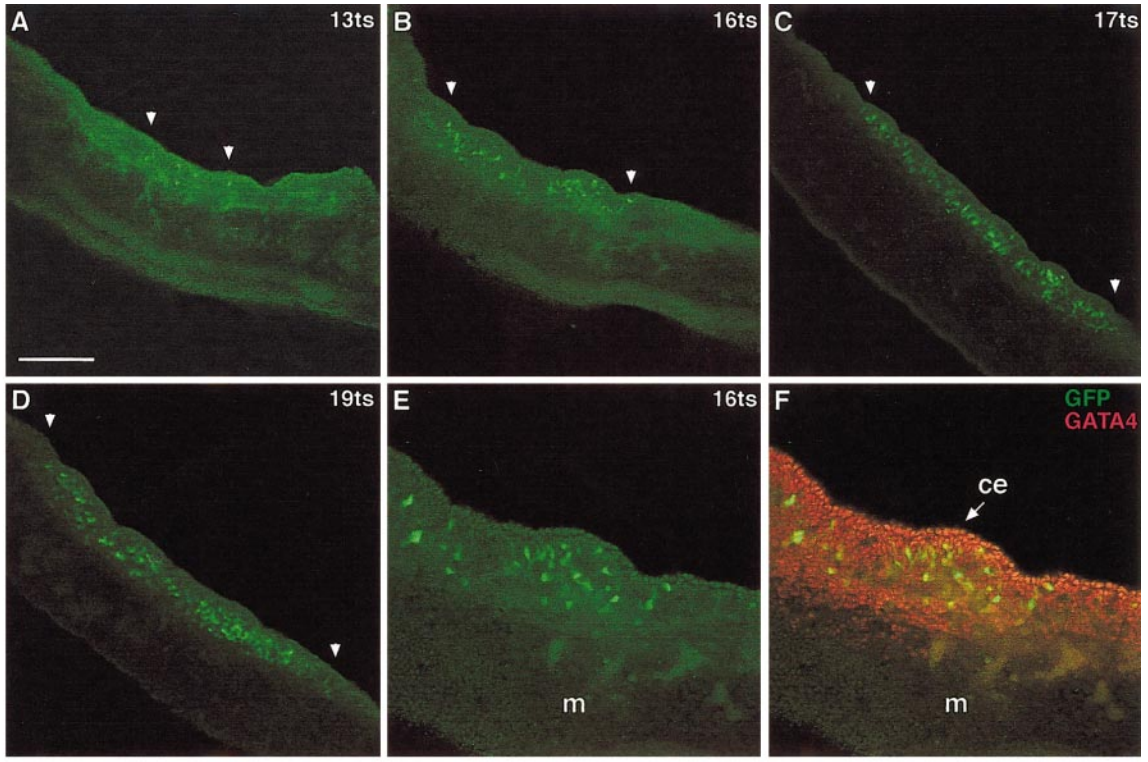
FIG. 1. The *Sry*-EGFP transgene construct (non-sex-reversing) compared to the 14.6-kb *Sry* transgene construct (sex-reversing) upon which it is based. The *Sry* promoter from the 14.6-kb construct was inserted upstream of the EGFP gene in pEGFP-1 (Clontech) which contains SV40 poly(A) signals. GRC indicates the glutamine repeat clusters. The *Sry* transcriptional start site and translational start (ATG) and stop (TAG) sites are indicated. All numbering refers to GenBank entry X67204.

FIG. 2. Confocal images of E11 XX (18ts) and XY (21ts) gonads prior to testis cord development showing EGFP expression (green) exclusively in gonadal somatic cells. GATA4 (red) is expressed in the nucleus of somatic cells and PECAM-1 (blue) is expressed on the surface of germ and vascular endothelial cells. EGFP-positive cells also express GATA4 but not PECAM-1. At this stage, XX (E, F) and XY (A–D) gonads appear identical. EGFP-positive cells have a mesenchymal morphology and are associate with and occasionally surround germ cells (asterisks in A and D). The mesonephros is not visible in the focal plane shown in (A–D) and is indicated by "m" in (E) and (F). Scale bar, 50 μm.

positive cells located in the center of the gonad with no cranial or caudal bias, 12 had EGFP-positive cells located in the center of the gonad with a caudal bias, and 2 had EGFP-positive cells located in the center of the gonad with a cranial bias.

EGFP-expressing cells were often clustered, suggesting they were dividing (Figs. 2A, 2E, and 3). Also, as early as the 19ts stage, EGFP-positive cells were closely associated with and sometimes partially surrounded individual germ cells (Figs. 2D and 2F).



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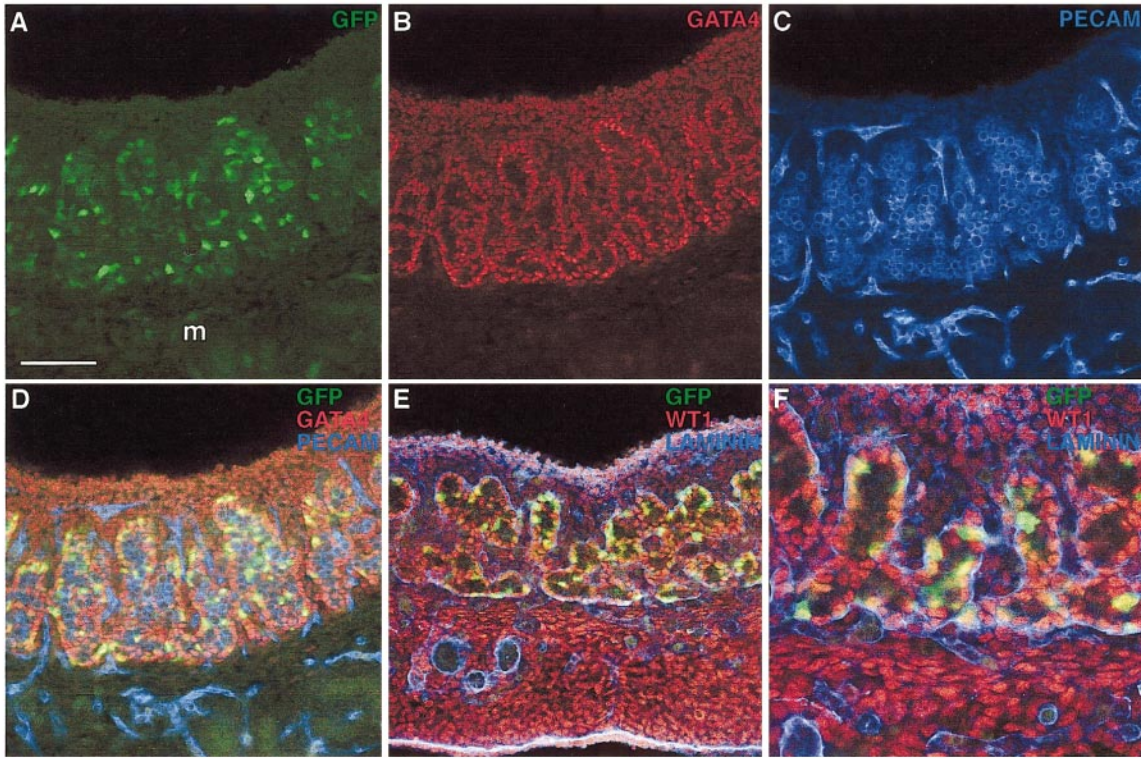

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FIG. 3. Confocal images of E11–E11.5 XY genital ridges between 13 and 19ts. *Sry* is first expressed in cells located centrally in the genital ridge and then in cells located at the poles (A–D). The arrowheads in (A–D) delimit the domain of *Sry*-EGFP expressing cells. (A–D) Virtually the entire genital ridge is shown. In these XY samples, *Sry*-EGFP expression was detected with an anti-GFP antibody (green). In all panels, the cranial pole is at the lower right corner and the caudal pole is at the upper left corner. The mesonephric component ("m" in E and F)

TABLE 1
Primary Antibodies

Antibody	Type	Antigen	Cell type identified	Source (reference)	Dilution
GFP	rabbit polyclonal	green fluorescent protein	transgene expressing	Molecular Probes	1:3000
AMH	rabbit polyclonal	Anti-müllerian hormone (Müllerian inhibiting substance)	Sertoli cells	N. Josso (Rey *et al.*, 1996)	1:250
GATA4 (C-20)	goat polyclonal	GATA-binding protein 4	gonadal somatic cells	Santa Cruz Biotechnology	1:500
WT1 (C-19)	rabbit polyclonal	Wilm's Tumor-1	gonadal and mesonephric somatic cells	Santa Cruz Biotechnology	1:50
WT1 (6F-H2)	mouse monoclonal	Wilm's Tumor-1	gonadal and mesonephric somatic cells	Dako	1:300
DMRT1	rabbit polyclonal	doublesex and mab-3 related transcription factor 1	germ and gonadal somatic cells (stage dependent)	V. Bardwell and D. Zarkower (Raymond *et al.*, 2000)	1:2000
CD31	rat monoclonal	platelet endothelial cell adhesion molecule	germ and vascular endothelial cells	BD PharMingen	1:100
SSEA-1 (MC-480)	mouse monoclonal	SSEA-1	germ cells	Developmental Studies Hybridoma Bank (Solter and Knowles, 1978)	1:50
LAMININ	rabbit polyclonal	laminin	basal lamina	Sigma	1:3000
CD49f	rat monoclonal	integrin α6 subunit	various	Serotec	1:2000
NCAM (5B8)	mouse monoclonal	neural cell adhesion molecule	various	Developmental Studies Hybridoma Bank (Dodd *et al.*, 1988)	1:10
NGFR	rabbit polyclonal	p75 nerve growth factor receptor	various	Chemicon	1:500

EGFP Expression during the Initial Morphological Divergence of XX and XY Gonads: E12–E15.5 (Beginning at the 24ts Stage)

The first obvious morphological sign of sex determination in mice is the initiation of testis cord differentiation in XY genital ridges at about E12. In the studies presented here, cord development was first evident in XY gonads from 22 to 23ts fetuses and nascent testis cords were first visible at ~24ts, as indicated by the alignment of Sertoli and germ cells and the presence of a basal lamina. Concurrently, the vasculature began to surround the nascent cords. Testis cord development probably is initiated when migrating mesonephric cells (primordial peritubular myoid cells) come into contact with pre-Sertoli–germ cell aggregates. Sertoli and peritubular myoid cells then cooperate to deposit the basal lamina. This process results in a very distinct architecture wherein Sertoli and germ cells are aligned within cords surrounded by a basal lamina with peritubular myoid cells surrounding the cords outside the basal lamina.

XY gonads. In XY gonads, starting at 24ts, EGFP-positive cells now were located inside nascent cords outlined by LAMININ. These cells continued to express WT1 and GATA4, and did not express PECAM-1 (Figs. 4D–4F). Also, EGFP-expressing cells were now absent from the developing tunica region (Figs. 4D and 4E). Coincident with

of the genital ridge is toward the lower left corner and the coelomic epithelium ("ce" in F) is toward the upper right. *Sry*-EGFP is not expressed in mesonephric cells nor is GATA4 (red). EGFP-expressing cells were not observed in the coelomic epithelium (E, F). However, at the earliest stages, EGFP-positive cells were present just below the coelomic epithelium and more were present nearer to the coelomic epithelium than to the mesonephros. Scale bar: A–D, 200 μm; E and F, 100 μm.

FIG. 4. Confocal images of E12 XY gonads at the beginning of testis cord development. (A–D) 25ts. *Sry* (GFP, green) is expressed in somatic cells (GATA4, red), not germ cells (PECAM-1, blue), within nascent cords. (E, F) 27ts. *Sry* (GFP, green) is expressed in somatic cells that also express WT1 (red) within nascent cords surrounded by a basal lamina (LAMININ, blue). WT-1 also is expressed in mesonephric cells (E, F). Pre-Sertoli cells are the only somatic cells within cords, therefore *Sry* is expressed in pre-Sertoli cells. The expression of GATA4 and WT1 (within the developing gonad) is increased, relatively, in pre-Sertoli cells coincident with cord development. *Sry*-EGFP-positive cells are no longer present outside of cords once cord development is evident (D–F). The gonad is at the top and the mesonephros (m) is at the bottom in each panel. Scale bar: A–E, 100 μm; F, 50 μm.

cord development, WT1 and GATA4 became more abundant in Sertoli versus interstitial cells as indicated by increased relative fluorescence (Figs. 4B, and 4D–4F). EGFP expression was down-regulated coincident with cord development so that at later stages (~27ts) the number of cells expressing EGFP and the intensity of the autofluorescence were greater where cords were less developed. Once cord development was initiated, the EGFP-positive cells became epithelial in morphology (Figs. 4F and 5D).

At E13.5–E14.5, testis cords were well developed and completely enclosed by a basal lamina. EGFP autofluorescence was greatly reduced and often difficult to distinguish from background, but EGFP was detectable by using the anti-GFP antibody. All EGFP-expressing cells were clearly within cords, were located adjacent to the basal lamina, continued to express GATA4 and WT1, and were PECAM-1 negative (Figs. 5C and 5D). Many EGFP-positive cells possessed cytoplasmic processes that extended to and partially surrounded germ cells (Fig. 5D, arrowheads). The expression of anti-Müllerian hormone (AMH; also known as Müllerian-inhibiting substance, MIS) is one of the earliest differential markers for Sertoli cells. AMH expression thus was examined to further ensure identification of the *Sry*-EGFP-expressing cells (Fig. 6). In E13.5 XY gonads, all *Sry*-EGFP-positive cells coexpressed AMH. However, not all AMH-expressing cells coexpressed *Sry*-EGFP. No EGFP- or AMH-positive cells coexpressed PECAM-1, indicating they were not germ cells.

The expression of a number of additional markers was examined in E12–E13.5 XY gonads, including NGFR, and DMRT1. NGFR, which is expressed in the precursors of peritubular smooth muscle cells (Campagnolo *et al.*, 2001), was examined in four gonads from E12 XY fetuses (25ts) in which testis cord development was evident (Fig. 7B). NGFR-expressing cells were present in the gonads throughout the region subadjacent to the coelomic epithelium and at the gonad/mesonephros boundary. In the center of the gonad, NGFR-expressing cells were more spatially restricted and were clearly outside of the developing cords. EGFP and NGFR expression did not overlap, indicating that EGFP-positive cells are not precursors of peritubular smooth muscle cells.

DMRT1 is required for testis differentiation and is expressed in a sexually dimorphic pattern in fetal gonads (Raymond *et al.*, 1999; Raymond *et al.*, 2000). At E13.5, DMRT1 was highly expressed in both germ and Sertoli cells, but was more abundant in Sertoli cells (Fig. 7D). Very weak expression also was observed in interstitial cells. We conclude that the EGFP-expressing cells have all the morphological characteristics of pre-Sertoli cells and express the appropriate marker proteins.

At E12.5–E14.5, EGFP expression was observed in most, but not all, pre-Sertoli cells (Figs. 4D, 4F, 5C, and 5D). Also, the intensity of EGFP autofluorescence and GFP antibody staining was variable. By E15.5, EGFP was barely detectable by using the GFP antibody (Fig. 8D). In fact, only a few cords contained cells with detectable EGFP and then expression was weak. However, it was clear that the EGFP-expressing cells did not express PECAM-1 and therefore they were not germ cells.

XX gonads. Between E12 and E15.5, the morphological differentiation of XX gonads is not nearly as apparent as in XY gonads. However, germ cells are mitotic through E13.5 and are present in clusters by E13.5–E14.5. Also, the ovarian vasculature is clearly developing at E13.5.

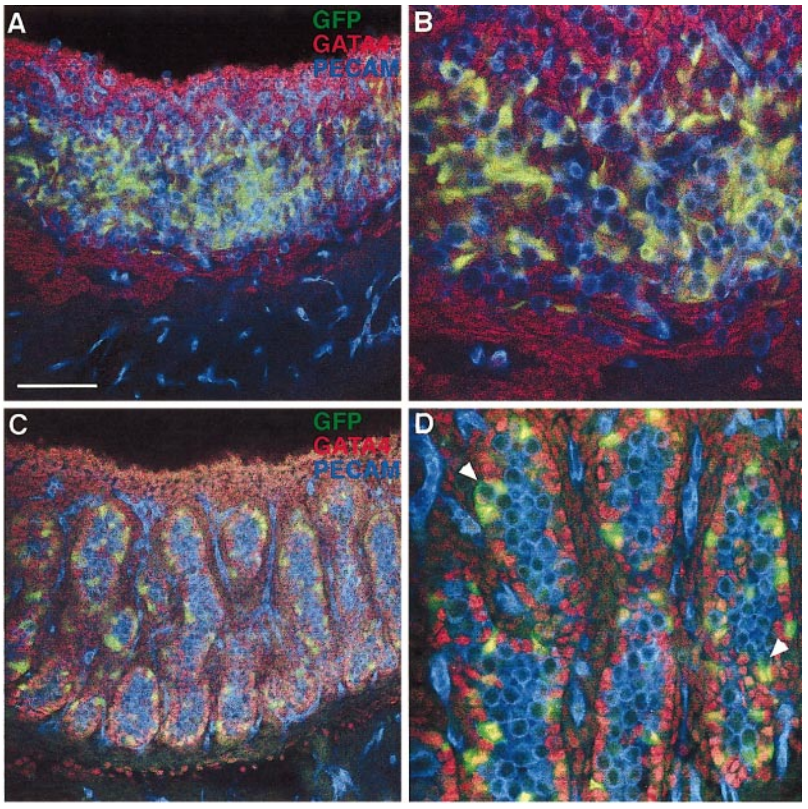
In E12.5–E13.5 XX gonads, EGFP-expressing cells continued to be WT1- and GATA4-positive and PECAM-1-negative, and tended to associate with germ cell clusters (Figs. 5A and 5B). However, GATA4 expression began to decrease in XX gonads at E13.5. A few EGFP-expressing cells surrounded individual germ cells. By late on day 12 (32ts), EGFP-expressing cells were localized closer to the mesonephros than the coelomic epithelium (Fig. 5A). Also, more EGFP-expressing cells were present in XX gonads than in XY gonads and EGFP autofluorescence remained easily detectable (Figs. 5A and 5C). The morphology of EGFP-expressing cells in XX gonads was mesenchymal through E15.5 (Figs. 5B and 8C).

At E14.5–E15.5, EGFP-expressing cells were localized to the central medullary region of the gonad (Figs. 8A and 8B). However, germ cells and well-developed vasculature were present throughout. Many individual germ cells within germ cell clusters were completely surrounded by EGFP-expressing cells (Fig. 8C). More EGFP-expressing cells were present in the interior of the gonad than near the surface.

The expression of a number of additional markers, including NGFR and DMRT1, was examined in gonads from XX transgenic E12–E13.5 fetuses. The expression of EGFP

FIG. 5. Confocal images of E13 (32ts) XX and XY gonads. Once testis cords begin to develop in XY gonads, the morphology of XX and XY gonads rapidly diverges as does the morphology of EGFP-expressing cells per se. (A, B) XX gonad. (C, D) XY gonad. The EGFP-positive pre-Sertoli cells in XY gonads are all within cords and have become epithelial in morphology. The EGFP-positive cells in XX gonads remain mesenchymal in morphology and are found mostly toward the mesonephros. Also, there are more EGFP-positive cells in XX gonads than in XY gonads. However, EGFP-expressing cells surround germ cells in both XX and XY gonads (arrowheads in D). Scale bar: A and C, 100 μm; B and D, 50 μm.

FIG. 6. Confocal images of an E13.5 XY gonad immunostained for EGFP (green), AMH (red), and PECAM-1 (blue) expression. Part of a single cord with surrounding vasculature is visible and is outlined with a dotted line in (D). All EGFP-expressing cells coexpress AMH (arrowheads in D) and therefore are pre-Sertoli cells. The autofluorescent cells outside of the cord in (A) are red blood cells (asterisks). Scale bar, 25 μm.



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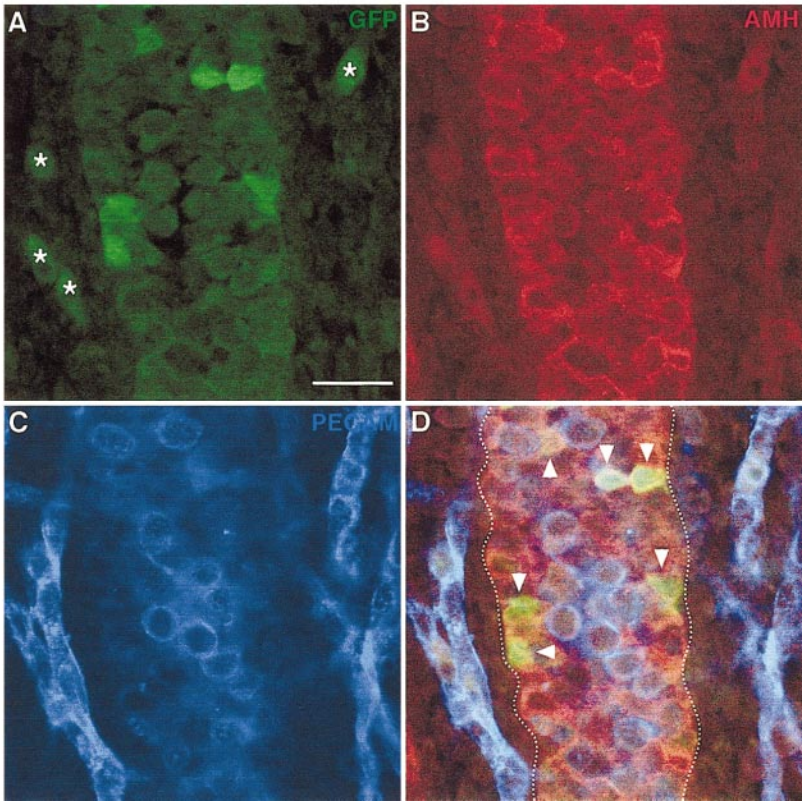

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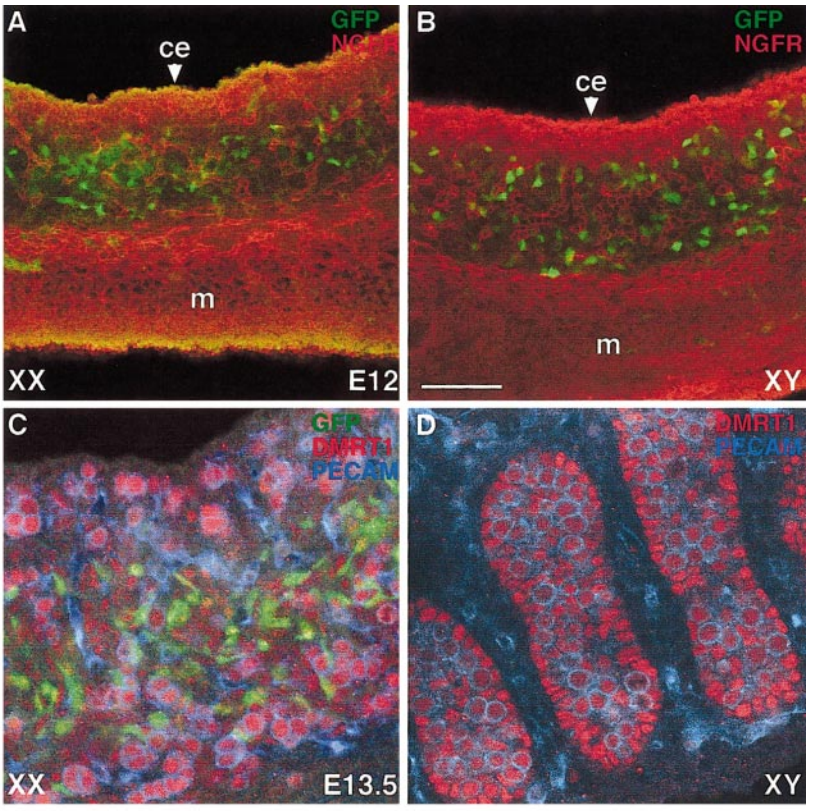

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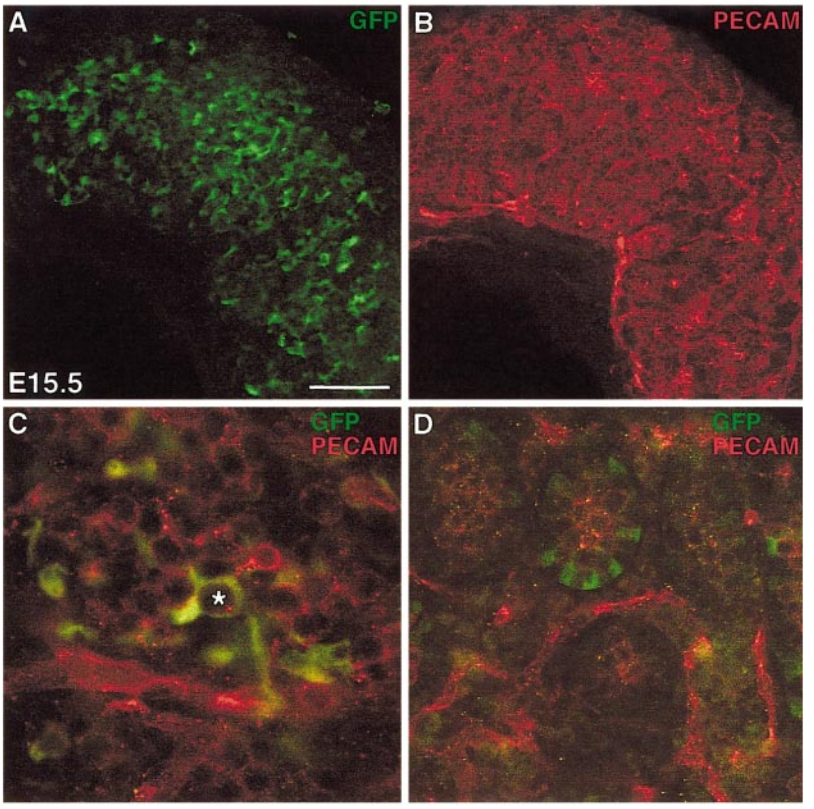

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and NGFR was examined in one pair of E12 (24ts) XX urogenital ridges (Fig. 7A). NGFR was expressed in both the mesonephros and gonad. In the gonad, NGFR was expressed in most of the cells adjacent to the mesonephros and coelomic epithelium. However, in the center of the gonad, expression was restricted to a subset of cells. EGFP-expressing cells also were present in the center of the gonad; however, EGFP and NGFR expression did not overlap.

DMRT1, PECAM-1, and EGFP expression was examined in one pair of gonads with attached mesonephroi from an E13.5 XX fetus (Fig. 7C). DMRT1 was present in both somatic and germ cells but was most abundant in germ cells. Some EGFP-expressing somatic cells also expressed DMRT1, but the EGFP-expressing cells tended to be in regions with the weakest DMRT1 expression.

EGFP Expression during Late Gestation and Early Postnatal Development: E18.5–P5

XY gonads. Four testes from E18.5 fetuses were examined for EGFP expression. Anti-GFP antibody staining was detectable only in two of four testes examined and then only very weakly in a few cells within some testis cords (data not shown). EGFP expression was not detectable by the anti-GFP antibody in three testes from P1 males.

XX gonads. Ovarian folliculogenesis begins at about E18.5 when a few primordial follicles with a single cell layer of presumptive granulosa cells surrounding individual germ cells can be identified. By P1, primordial follicles are more abundant and a basal lamina is often present surrounding the pregranulosa cells and oocyte. A few days later, at P5, more-advanced stage follicles are present.

Two ovaries from E18.5 fetuses were examined for EGFP expression. In contrast to XY gonads, EGFP expression was readily detectable by using the GFP antibody. EGFP-expressing cells were concentrated in the medulla and often surrounded single germ cells (oocytes). In a few primordial follicles, EGFP expression was detected in presumptive granulosa cells (Fig. 9A).

EGFP expression also was examined in postnatal ovaries. EGFP autofluorescence was not detectable in whole-mount ovaries from P1 females, However, GFP antibody staining was detectable in pregranulosa cells of primordial follicles (Fig. 9B). Cryostat sections of P5 ovaries were examined to more clearly define the EGFP-expressing cells (Figs. 9C–9F). At this time, most follicles are surrounded by a basal lamina that is secreted by the granulosa cells. Only a few EGFP-positive cells were observed and all were somatic cells within follicles surrounded by a basal lamina. Because pregranulosa cells are the only somatic cells within follicles, we conclude that Tg92 is expressed in pregranulosa cells in XX gonads.

DISCUSSION

Summary

The mammalian gonad begins development as an undifferentiated, bipotential anlagen known as the genital ridge. The expression of *Sry* in the genital ridges of XY fetuses initiates testis development and is hypothesized to do so by directing supporting cell precursors to develop as Sertoli cells and not as granulosa cells (Burgoyne *et al.*, 1988a). To directly test this hypothesis, transgenic mice expressing EGFP under the control of the *Sry* promoter were produced. After establishing that the transgene was expressed in fetal gonads at the time when endogenous *Sry* is expressed, the spatial and temporal expression of *Sry*-EGFP in developing gonads was examined by using confocal microscopy and immunofluorescent histochemistry. This analysis indicated that: (1) *Sry* is expressed in gonadal somatic cells and not in either germ cells or cells within the adjacent mesonephros, (2) *Sry* is first expressed in cells located internally and centrally in the genital ridge and then later in cells located near the surface and at the cranial and caudal poles (with a bias toward the caudal pole), (3) *Sry* is expressed in pre-Sertoli cells in the testis, and (4) the *Sry*-EGFP transgene is expressed in pregranulosa cells in the ovary.

We conclude that *Sry* is expressed in pre-Sertoli cells and that Sertoli and granulosa cells develop from a common precursor. These results support the hypothesis that *Sry* initiates testis differentiation by directing the development of supporting cell precursors as Sertoli rather than granulosa cells. Furthermore, we present a hypothesis based on

FIG. 7. Confocal images of XX and XY gonads immunostained with additional markers. (A, B) XX (A) and XY (B) E12 gonads (25ts) immunostained for NGFR (red) expression. In both sexes, NGFR is expressed in most cells near the coelomic epithelium (ce) and the mesonephros (m), but is expressed only in a subset of cell in the middle of the gonad. In XY gonads, the NGFR-expressing cells are outside of the developing cords. EGFP-positive cells do not express NGFR in either sex. (C, D) XX (C) and XY (D) E13.5 gonads immunostained for DMRT1 (red) and PECAM-1 (blue). (Only the gonad is visible.) In both sexes, DMRT1 is expressed in somatic and germ cells; however, expression is higher in germ cells in the female and higher in pre-Sertoli cells in the male. DMRT1 expression is essentially confined to cells within cords in XY gonads. In the female, EGFP expression partially overlaps DMRT1 expression in somatic cells. Scale bar: A and B, 100 μm; C and D, 50 μm.

FIG. 8. Confocal images of E15.5 XX (A–C) and XY (D) gonads. (A, B) In XX gonads, EGFP expression remains strongly detected with the anti-GFP antibody and is localized to cells within the medulla of the developing ovary. (C) EGFP-positive cells often surround individual germ cells within germ cell clusters (asterisk). (D) In contrast, EGFP expression is only weakly detected in Sertoli cells in the developing testis. Scale bar: A and B, 200 μm; C and D, 50 μm

the *Sry* expression pattern to explain the nonrandom distribution of testicular and ovarian tissue in mammalian ovotestes.

Sry-EGFP Transgene as a Reporter for Sry Expression

Because the *Sry*-EGFP transgene was designed to be non-sex-reversing so that its expression could be analyzed in developing ovaries and testes, sex reversal of XX transgenic mice could not be used to indicate that the *Sry*-EGFP transgene was expressed in an appropriate manner. Therefore, it was important to establish that Tg92 was expressed in a manner similar to endogenous *Sry*. In the mouse, *Sry* is expressed in XY genital ridges between about E10.5 and E13 with peak RNA expression at E11.5. Tg92 was transcribed at E10.5, transcript levels increased at E11.5, and decreased thereafter. However, transgene transcription was not completely extinguished and low transcript levels were present at E15.5, P1, and P28 in XX and XY gonads. This result is not surprising given that other *Sry* transgenes that contain the same promoter region and cause XX sex reversal have a similar expression pattern (Bergstrom *et al.*, 2000). We suggest that transgenes based on the 14.6-kb *Sry* construct lack a *cis*-acting regulatory element necessary for transcriptional silencing.

The expression of EGFP, as measured by autofluorescence and anti-GFP antibody detection, also indicated Tg92 was expressed properly. As noted previously, the fluorescent signal intensity was greater using the anti-GFP antibody and indirect immunofluorescence than for EGFP autofluorescence. Over developmental time, EGFP expression was initially undetectable, was first detected only with the anti-GFP antibody, was subsequently visible by autofluorescence, and later was detected only with the anti-GFP antibody. This pattern indicates transgene expression is first increased and then decreased. More importantly, in XY gonads, EGFP autofluorescence was clearly visible for only a short period between ~E11.5 and E13. This timing is significant because it agrees with what is predicted from previously established *Sry* mRNA expression patterns (assuming sufficient EGFP needs to accumulate before it is visible and taking into account that EGFP is a very stable protein). Furthermore, in agreement with previous data, EGFP expression was not detected in the mesonephros or developing metanephric kidney. We conclude the *Sry*-EGFP transgene is correctly regulated in XY gonads and is an accurate reporter of *Sry* expression. This conclusion is further supported by recent RNA *in situ* hybridization results (Bullejos and Koopman, 2001) as discussed below.

We are intrigued by the finding that EGFP expression persisted at a high level in females well after expression was down-regulated in males. A number of explanations are possible, the most compelling being that a male-specific gene is involved in silencing *Sry* expression. This gene could be *Sry* itself or a gene downstream of *Sry*. This idea is supported by the observation that, in XY gonads, EGFP autofluorescence began to diminish coincident with testis cord development. Also, it is interesting to note the transgene was up- and down-regulated similarly to endogenous *Sry* despite the fact it contained a heterologous 3′ UTR.

Sry Is Expressed First in Cells Located Centrally in the Genital Ridge and Then Later in Cells at the Cranial and Caudal Poles

Previous *in situ* hybridization experiments indicated that *Sry* might be expressed in a cranial-to-caudal wave (Swain *et al.*, 1998). However, the observations presented here, which are consistent with those independently reported by Bullejos and Koopman (2001), do not support this assertion. If *Sry* was expressed in a wave propagated from the cranial-to-caudal pole, then we would have observed gonads with EGFP-expressing cells present only at the cranial or caudal pole. This was not our finding. In fact, EGFP expression began centrally and was only later observed in cells at the cranial and caudal poles. This pattern indicates *Sry* is expressed first in the center of the gonad and then spreads anteriorly and posteriorly. It is satisfying to note that nearly twenty years ago it was predicted that *Sry* (called *Tdy* at that time) would be expressed first in the central region of the gonad and then spread toward the cranial and caudal ends (Eicher, 1982).

The center-to-pole *Sry* expression pattern provides an explanation for the nonrandom distribution of testicular and ovarian tissue in mammalian ovotestes. An ovotestis is a gonad composed of both ovarian and testicular tissue. Mouse ovotestes, regardless of what caused the aberrant gonadal development, usually develop with testicular tissue present in the center of the gonad and ovarian tissue present at the poles (E.M.E. *et al.*, in preparation). Occasionally, one pole will develop as ovarian tissue, while the other develops as testicular tissue. In this case, there is a bias toward testicular tissue development at the caudal pole. Ovotestes with ovarian tissue present in the central region flanked by testicular tissue at the poles are extremely rare (Eicher *et al.*, 1980). And, to our knowledge, ovotestes with interspersed ovarian and testicular tissue have not been reported. The *Sry* expression pattern suggests that, in mice, testicular tissue begins to develop first in the center of the gonad. Therefore, if *Sry* expression is weak and/or interrupted, testicular tissue would develop preferentially in the center of the gonad, as is observed in ovotestes. The findings reported here as they related to ovotestis development are more fully discussed in Eicher *et al.* (in preparation). An interesting unanswered question is what causes the initiation of *Sry* expression in the center of the gonad.

Sry Is Expressed in Pre-Sertoli Cells

Although previous experimental data suggested that *Sry* is expressed in pre-Sertoli cells, direct evidence was lacking (Burgoyne *et al.*, 1988a; Patek *et al.*, 1991; Rossi *et al.*, 1993;

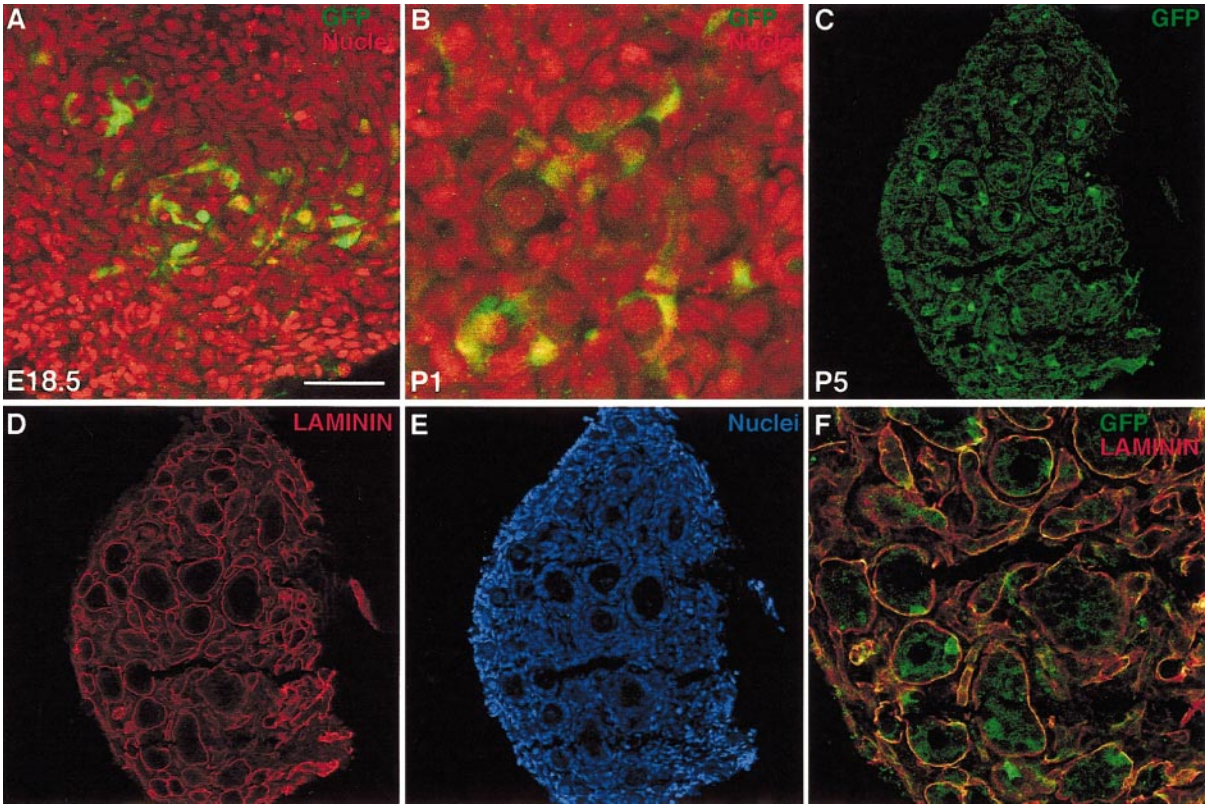

FIG. 9. Confocal images of developing E18.5 (A), P1 (B), and P5 (C–F) ovaries. (A, whole-mount) In E18.5 ovaries, a few primordial follicles are developing and some presumptive pregranulosa cells surrounding oocytes are EGFP-positive. (B, whole-mount) In P1 ovaries, primordial follicles are more fully developed and some pregranulosa cells surrounding oocytes (large round nuclei) are EGFP-positive. (C–E, cryostat sections) Follicles of more advanced stages are now present and surrounded by a basal lamina (LAMININ, red). Pregranulosa cells within enveloped follicles are positive for EGFP expression. The oocytes in (F) are represented by the circular black (nonstaining) region in the center of each follicle. Scale bar: A and F, 50 μm; B, 25 μm; C–E, 100 μm.

Swain *et al.*, 1998; Salas-Cortes *et al.*, 1999). The expression pattern of the *Sry*-EGFP transgene now provides direct evidence for *Sry* expression in pre-Sertoli cells. At all stages examined, EGFP expression was detected in gonadal somatic cells and not in germ cells or cells in the adjacent mesonephros. This finding is consistent with previous data (Koopman *et al.*, 1990; Rossi *et al.*, 1993; Jeske *et al.*, 1995). The evidence that EGFP (i.e., *Sry*)-expressing cells in XY gonads are pre-Sertoli cells is as follows. EGFP-expressing cells were: (1) associated with and occasionally surrounded germ cells as soon as EGFP expression was detectable in undifferentiated gonads, (2) present exclusively within developing testis cords as soon as cords were identifiable, (3) identified as somatic cells within well-developed cords surrounded by a basal lamina, and (4) identified as cells that coexpressed AMH at E13.5. Based on the above evidence and because Sertoli cells are the only somatic cells within testis cords, we conclude *Sry* is expressed in pre-Sertoli cells.

Lineage-tracing experiments in mice established that the coelomic epithelium contributes cells to the Sertoli and interstitial cell lineages (Karl and Capel, 1998). However, the ability of cells from the coelomic epithelium to become Sertoli cells was developmentally restricted to stages prior to 18–20ts. In the experiments presented here and those recently reported by Bullejos and Koopman (2001), *Sry*-EGFP expression was not detected in cells within the coelomic epithelium, which may indicate sufficient EGFP had not accumulated within coelomic epithelium cells to be detectable. However, it also is possible that migrating coelomic epithelium cells do not express *Sry* until they reach a more internal location within the genital ridge, an idea supported by the observation that cells from the coelomic epithelium also migrate into XX gonads (Karl and Capel, 1998). The lineage-tracing experiments do not exclude the possibility that the mesonephros also contributes cells to the Sertoli cell lineage. However, coculture experiments in mice indicated that, if the mesonephros donates cells to the Sertoli cell lineage, it must do so before E11.5 (Martineau *et al.*, 1997). In this regard, it is interesting to note that EGFP-expressing cells were not detected in the mesonephros and EGFP-expressing cells were first detected

proximal to the coelomic epithelium. However, the data presented here do not exclude the possibility that mesonephric cells, which migrated before *Sry* is expressed, contribute to the pre-Sertoli cell population.

Once cords began to develop so that pre-Sertoli cells could be identified definitively, EGFP expression was not detected in all pre-Sertoli cells. It is possible this result is an experimental artifact attributable to variegated transgene expression. However, it also is possible that this result represents the endogenous *Sry* expression pattern. For example, a given pre-Sertoli cell might only express *Sry* for a short period of time and *Sry* expression may not be synchronized among pre-Sertoli cells. Furthermore, once the Sertoli cell fate is initiated by *Sry* expression, continued *Sry* expression in decendents of pre-Sertoli cells may not be necessary. Another possibility is that *Sry* expression is not an absolute requirement for Sertoli cell differentiation. In fact, experimental data support this possibility because, although most Sertoli cells were XY in opposite sex chimeras, some were XX (Palmer and Burgoyne, 1991; Patek *et al.*, 1991). Thus, there may be an *Sry*-independent period when *Sry*-expressing pre-Sertoli cells are able to locally recruit *Sry*-nonexpressing cells into the Sertoli cell pathway. Additional experiments are needed to distinguish among these possibilities.

Sertoli and Granulosa Cells Share a Common Precursor

There is significant structural and functional evidence to suggest that Sertoli and granulosa cells develop from a common precursor (reviewed in McLaren, 1991). They are considered to be homologous cells because both are epithelial cells that "nurse" germ cells and are confined within compartments. Furthermore, in opposite sex chimeras, granulosa cells are predominately XX (thus *Sry*-nonexpressing), whereas Sertoli cells are predominately XY (thus *Sry*-expressing) (Burgoyne *et al.*, 1988b; Patek *et al.*, 1991). We reasoned that if Sertoli and granulosa cells develop from a common precursor, expression of the non-sex-reversing *Sry*-EGFP transgene in XX gonads would mark the cell lineage fated to become granulosa cells. The evidence that EGFP-expressing cells in XX gonads were pregranulosa cells is as follows. EGFP-expressing cells: (1) were associated with and occasionally surrounded germ cells as soon as EGFP expression was detectable in undifferentiated gonads, (2) often surrounded individual germ cells within germ cell clusters at later stages, (3) were closely associated with oocytes when primordial follicles were first developing, and (4) were identified as somatic cells within follicles surrounded by a basal lamina. Therefore, we conclude that in XX gonads the *Sry*-EGFP transgene is expressed in pregranulosa cells. This result, especially when combined with previous structural and functional evidence, provides compelling evidence that Sertoli and granulosa cells share a common precursor.

During differentiation, pre-Sertoli and pregranulosa cells undergo a mesenchymal-to-epithelial transition. Because Sertoli cells differentiate in XY gonads a number of days before granulosa cells differentiate in XX gonads, they undergo this transformation first. In fact, Sertoli cell differentiation and epithelial transformation is essentially coincident with the development of testis cords. On the other hand, granulosa cells undergo this mesenchymal-to-epithelial transition coincident with the maturation of follicles. In undifferentiated XX and XY gonads, the EGFP-expressing cells shared a similar mesenchymal morphology. Once testis cords begin to develop in XY gonads, the EGFP-expressing cells assumed an epithelial morphology. During this same time in XX gonads, the EGFP-expressing cells remained mesenchymal in morphology and remained so until follicles began to develop.

As noted previously, lineage-tracing experiments showed that cells from the coelomic epithelium migrate into XX gonads (Karl and Capel, 1998). However, because appropriate markers were not available, the fate of these cells could not be determined. Although the finding that Sertoli and granulosa cells develop from a common precursor argues granulosa cells also originate from cells derived from the coelomic epithelium, it would be interesting to repeat the cell lineage-tracing experiments in gonads from fetuses carrying the *Sry*-EGFP transgene.

To our knowledge, the *Sry*-EGFP transgene (Tg92) provides the earliest differential marker of the supporting cell lineage in XX and XY gonads and should be valuable in a number of future experiments.

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