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Wt1 functions in the development of germ cells in addition to somatic cell lineages of the testis

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Abstract

The Wilms' tumor suppressor gene, *Wt1*, encodes a transcription factor critical for development of the urogenital system. To identify lineages within the developing urogenital system that have a cell-autonomous requirement for *Wt1*, chimeric mice were generated from *Wt1*-null ES cells. Males with large contributions of *Wt1*−/− cells showed hypoplastic and dysgenic testes, with seminiferous tubules lacking spermatogonia. *Wt1*-null cells contributed poorly to both somatic and germ cell lineages within the developing gonad, suggesting an unexpected role for *Wt1* in germ cell development in addition to a role in the development of the somatic lineages of the gonad. *Wt1* expression was detected in embryonic germ cells beginning at embryonic day 11.5 after migrating primordial germ cells (PGCs) have entered the gonad. Germ cells isolated from *Wt1*-null embryos showed impaired growth in culture, further demonstrating a role for *Wt1* in germ cell proliferation or survival. Therefore, *Wt1* plays important, and in some cases previously unrecognized, roles in multiple lineages during urogenital development.

Keywords: *Wt1*; Germ cells; Gonadal development

Introduction

The Wilms' tumor suppressor gene *Wt1* encodes a zinc-finger-containing nuclear protein that is critical for urogenital development and function (Call et al., 1990; Gessler et al., 1990; Kreidberg et al., 1993). Mutations in this gene are associated with three distinct syndromes in humans: WAGR syndrome, Denys–Drash syndrome, and Frasier syndrome (reviewed by Scharnhorst et al., 2001). The common features of these syndromes are predisposition to kidney failure and defects in reproductive system development suggestive of impaired masculinization. Mice completely lacking *Wt1* function demonstrate complete gonadal agenesis; initial development of the gonadal ridge occurs in these animals but subsequent development is arrested (Kreidberg et al., 1993). Mice with mutations that affect splicing of a three amino acid sequence (KTS) within the third zinc finger domain show developmental abnormalities of the gonad (Hammes et al., 2001). Homozygotes that are incapable of generating the +KTS isoform develop gonads that morphologically resemble ovaries in both XX and XY animals, and impaired expression of *Sry* was detected in XY animals. Homozygotes incapable of generating the KTS isoforms developed hypoplastic, poorly differentiated gonads. These results show the importance of *Wt1* for urogenital development.

Development of the gonad is a complex process that requires the function of many genes, including *Wt1* (for a review, see Capel, 2000; McLaren, 2000). *Wt1* is first

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detectable in the intermediate mesoderm, which gives rise to the urogenital ridge (Armstrong et al., 1993; Buckler et al., 1991; Pelletier et al., 1991). In the gonadal ridge and the indifferent gonad, *Wt1* expression is seen in both the coelomic epithelium and the underlying mesenchyme (Pelletier et al., 1991). Expression of *Wt1* is also seen in the adjacent mesonephros (Pelletier et al., 1991). In a normal animal, the coelomic epithelial cells overlying the gonadal ridge migrate into the gonadal ridge to become the Sertoli and granulosa cells populations of the male and female, respectively (Albrecht and Eicher, 2001; Karl and Capel, 1998). *Wt1* expression is maintained in Sertoli and granulosa cells in adults (Armstrong et al., 1993; Pelletier et al., 1991). In males, a population of mesonephric cells migrate into the developing gonad in response to Sertoli cell-derived signals and give rise to the peritubular and vascular endothelial cells (Capel et al., 1999; Martineau et al., 1997; Tilmann and Capel, 1999). The origin of the fetal Leydig cells is unclear but they are likely to differentiate from either migrating mesonephric cells or the gonadal mesenchyme, both of which are derivatives of the intermediate mesoderm (Brennan et al., 2003, and references therein). Peritubular cells are thought to cause a secondary wave of Leydig cells that appear in the prepubertal testes (Mendis-Handagama and Ariyaratne, 2001). Thus, all somatic cells of the fetal and adult gonad are presently thought to be derived from a *Wt1*-expressing population of cells in the intermediate mesoderm. The germ cells are the only lineage within the gonad that has not been previously demonstrated to express *Wt1* at some point during their development.

In mammals, primordial germ cells (PGCs) arise at the base of the allantois and migrate through the hindgut mesentery into the gonadal ridge (reviewed by Zhao and Garbers, 2002). A number of mutations can disrupt this process, including mutations that affect the c-kit or SCF pathway and β1-integrins (Anderson et al., 1999). Once the primordial germ cells enter the gonadal ridge, they lose their motility and begin to express markers such as GCNA and *Mvh* (Donovan et al., 1986; Enders and May, 1994; Toyooka et al., 2000). The gonadal germ cells are then induced to differentiate into the prospermatogonia and prooogonia in response to signals from the somatic supporting cells; in males, this process occurs as germ cells become incorporated into seminiferous tubules (Adams and McLaren, 2002). In *Wt1*-null embryos, germ cell formation and migration occurs normally, but development of the gonadal ridge is impaired (Kreidberg et al., 1993). Therefore, it is not possible to determine if *Wt1* plays later roles in germ cell development using these embryos.

Previous studies have demonstrated that *Wt1* is critical for gonadal development but have not examined the importance of *Wt1* function within the various cell lineages of the developing gonad. To identify those gonadal lineages that require *Wt1* cell autonomously for proper development or function, we generated chimeric mice by injecting *Wt1*-null ES cells into wild-type blastocysts. Here we show that *Wt1* is not only required for development of the somatic lineages, but is also expressed and required in the germ cell lineages of the developing gonad.

Materials and methods

Generation of *Wt1*-null ES cell lines: Two XY, *Wt1*-null ES cell lines with normal karyotypes were isolated, one by isolating ES cells de novo from *Wt1*-null embryos (Robertson, 1987) and the other by retargeting the remaining wild-type allele in the original J1-derived ES cell line used to generate *Wt1*-null mice (Kreidberg et al., 1993). A gene-targeting vector nearly identical to that originally used to delete the *Wt1* gene was generated by replacing the neomycin resistance gene with a hygromycin resistance gene. The genetic background of the resulting ES cell lines was either 129/SvJae for the J1 ES cell-derived cells or a mixed C57BL/6 and 129/SvJae background for the line derived from *Wt1*-null embryos. Both cell lines generate mice with an agouti coat color, allowing the percent chimerism to be estimated by coat color analysis when injected into host blastocysts with a black coat. The J1 ES cell-derived line is homozygous for the $Gpi1^a$ allele, while the ES cell line with a mixed genetic background carries both the $Gpi1^a$ and $Gpi1^b$ alleles; both of these alleles can be distinguished from the $Gpi1^{bb}$ alleles present in C57BL/6 mice using GPI isozyme analysis.

Generation of chimeric animals: Chimeric animals were generated by microinjecting C57BL/6 blastocysts with 10–15 *Wt1*-null ES cells as described by Bradley (1987), with the modifications described by Li et. al. (1992). GPI isozyme analysis was performed using standard procedures (Nagy and Rossant, 1993).

Histological analysis of chimeric animals: Tissues for paraffin embedding were fixed in 4% paraformaldehyde (PFA) overnight. Five micron sections were cut and stained with hematoxylin and eosin. Photographs were taken on a Nikon TE300 microscope equipped with a Spot Diagnostics digital camera.

XIST RT-PCR analysis: RNA was isolated from a single E13.5 hindlimb or tail as described (Chomczynski and Sacchi, 1987). First-strand cDNA was synthesized using the ProStar RT-PCR kit (Stratagene) as described by the manufacturer. First-strand cDNA was used directly in PCR reactions containing the primers 5′-TCTTCAAGTCCTCGCTACTC-3′ and 5′-CACACCCACAATACACACTC-3′, 1.5 mM $MgCl_2$, and ×1 Q-solution as recommended by the manufacturer (Qiagen). Cycling parameters were as follows: 93°C × 5′; 30 × [58°C × 1′; 72°C × 1.5′; 93°C × 1′]; and 72°C × 5′.

Immunohistochemistry: Immunohistochemistry was performed as described (Kreidberg et al., 1999). Antibodies were diluted in 10% goat serum in PBS + 0.1% Tween 20 as follows: anti-WT1 (sc-192; Santa Cruz Biochemicals) was used as described (Kreidberg et al., 1999); anti-smooth

muscle actin (alkaline phosphatase-conjugated; Sigma) was used at 1:200 and detected using BCIP and NBT (Roche Molecular Biochemicals).

In situ hybridization: Paraffin sections were processed for in situ hybridization with digoxygenin-labeled riboprobes synthesized with T7, T3, or SP6 RNA polymerase following the manufacturer's protocol (Roche Molecular Biochemicals). Hybridization was performed as described (Wilkinson and Nieto, 1993), with the following exceptions: prehybridization and hybridization was performed at 55°C and slides were washed twice for 30 min at 55°C in ×0.2 SSC for the high-stringency wash. After color development, slides were rinsed with PBS, fixed overnight with 4% PFA in PBS, and counterstained with eosin. The probes used in this study are as follows: *Wt1*, nt 435-1936 of mouse cDNA; *Sox9*, nt 423-1193 of mouse cDNA; *Cyp17a1*, nt 48-948 of mouse cDNA; *Dhh*, nt 16-770 of mouse cDNA; *Star*, nt 357-1432 of mouse cDNA; *Lhcgr*, nt 940-1860 of mouse cDNA; and *Kitl*, nt 1135-2016 of mouse cDNA. Photographs were taken on a Nikon TE300 microscope equipped with a Spot Diagnostics digital camera and processed with Adobe Photoshop 5.5.

Immunofluorescence and fluorescent alkaline phosphatase (AP) detection: Tissues were embedded for cryosectioning and 5-μm sections were processed for immunofluorescence as described (Watkins, 1992). For dual alkaline phosphatase or anti-laminin immunofluorescent labeling, cryosections were washed twice in AP buffer (100 mM Tris, 50 mM MgCl2, 100 mM NaCl, 0.1% Tween 20, pH 9.5), incubated at room temperature in AP buffer + 1 mg/ml Fast Red and 0.4 mg/ml Naphthol AS-MX (Sigma) for 1 h, rinsed twice in PBS + 0.1% Tween 20, postfixed for 15 min with 4% PFA in PBS, and processed for immunostaining. For immunofluorescent detection of WT1 and GATA4, paraffin sections were treated as described previously (Kreidberg et al., 1999), and minimally cross-reactive fluorescent secondary antibodies were used for detection. Antibodies were diluted in 10% goat serum in PBS + 0.1% Tween 20 as follows: rabbit anti-WT1 (Santa Cruz Biotechnology sc-192) at 1:200; goat anti-GATA4 (Santa Cruz Biotechnology sc-1237) at 1:200; Texas Red-conjugated donkey anti-rabbit IgG (minimal cross-reactivity; Jackson Immunoresearch) at 1:200; FITC-conjugated donkey anti-goat IgG (minimal cross-reactivity; Jackson Immunoresearch) at 1:200; rabbit anti-pan laminin antibodies (Sigma) at 1:200; and FITC-conjugated donkey anti-rabbit IgG antibody (Jackson Immunoresearch) at 1:200. Nuclei were counterstained with DAPI before photography.

Gonadal disaggregation and immunofluorescent labeling: Gonadal rudiments or neural tube fragments were isolated from wild-type mouse embryos, rinsed in PBS + 1 mM EDTA and disaggregated in PBS + 1 mM EDTA + 0.25% trypsin for 10 min at 37°C. Trypsinization was stopped by the addition of DMEM + 10% fetal calf serum and gonads were disaggregated by pipetting repeatedly. The resulting cell suspension was rinsed with PBS before DNase I treatment for 15 min at 37°C in PBS + calcium and magnesium (Mediatech) + 100 U/ml DNase I. Cells were washed once with PBS, then fixed for 15 min with 2% PFA in PBS. Immunofluorescent detection of SSEA-1 and WT1 was performed as described above. The antibodies for detection were as follows: SSEA-1, clone MC-480 (Developmental Studies Hybridoma Bank; 1:1 dilution); and WT1, sc-192 (Santa Cruz Biochemicals; 1:100 dilution).

Primordial germ cell isolation and RT-PCR: The trunk region or gonads were isolated from E10 or E12 unsexed Oct4-GFP transgenic embryos (Bortvin and Page, unpublished), respectively. A mixture of male and female PGCs were collected directly into TRIzol reagent using a Becton Dickinson cell sorter. Following collection, the TRIzol reagent was extracted twice with chloroform using Phase Lock Gel Heavy 0.5 ml tubes (Eppendorf). Total RNA was precipitated with an equal volume of isopropanol in the presence of 1.5 μl of GlycoBlue (Ambion). Residual genomic DNA was removed using DNase treatment (DNA-free reagents, Ambion). RNA was reprecipitated, washed with 70% ethanol, and dissolved in RNAse-free water (Ambion). First strand cDNA synthesis and universal amplification (four cycles) were performed using the SMART PCR cDNA synthesis kit (Clontech). cDNA was purified using QIAquick PCR purification kit (Qiagen), eluted in 100 μl of water. PCR was performed as described. Primers used for PCR amplification were as follows: *Wt1*, 5′-CCATTGAATGCATGAC-3′ and 5′-CGCAAACTTTTTCTGACAAC-3′; *Oct4*, 5′-TGTGGACCTCAGGTTGGACT-3′ and 5′-CTTCTGCAGGGCTTTCATGT-3′; *Gapd*, 5′-GTCATTGAGAGCAATGCCAG-3′ and 5′-GTGTTCCTACCCCCAATGTG-3′; and *Renin* 1, 5′-GTCTTTGACCACATTCTCTCC-3′ and 5′-CACAGCCTTCTTCACATAGC-3′. PCR products were resolved on 2% agarose gels.

Germ cell culture: Gonadal rudiments were isolated from F1 129/SvJae × C57BL/6 *Wt1*+/− females mated to 129Sv/Jae *Wt1*+/− males at E11.5; use of F1 females was necessary as 129/SvJae *Wt1*+/− females are infertile (Kreidberg et al., 1999). Gonadal rudiments were disaggregated as indicated above, and approximately 0.04 embryo equivalents were plated onto an irradiated STO/SNL1 feeder layer (ATCC) in Knockout DMEM (Invitrogen) + 15% ES qualified fetal calf serum (Hyclone) + penicillin–streptomycin–glutamine + 1% MEM nonessential amino acids (Invitrogen). Media were changed every 24 h. Cells were fixed for 15 min in 4% PFA in PBS before rinsing with AP buffer. Alkaline phosphatase activity was detected by incubation with 175 ng/ml BCIP and 75 ng/ml NBT (Roche Molecular Biochemicals) in AP buffer.

Preparation of gonadal somatic cell feeders: To generate germ-cell-depleted gonads for use as a feeder layer in germ cell cultures, F1 129/SvJae × C57BL/6 *Wt1*+/+ females mated to 129Sv/Jae *Wt1*+/+ males were intraperitoneally injected with 6.6 mg/g busulfan in a 1:1 H_2O–DMSO mix at E9.5; F1 females were used as a source of wild-type embryos to match the genotypes of the females used to

generate the *Wt1*−/− embryos. Gonads were harvested at E11.5 then disaggregated and cultured as described above.

Germ cell isolation: Gonads were isolated from individual E11.5 embryos harvested from WT1+/− × WT1+/− matings and incubated in PBS + 1 mM EDTA for 15 min at 37°C before liberating the germ cells by repeatedly poking the gonad with a 27-g needle as described (De Felici and McLaren, 1982). Approximately 0.04 embryo equivalents were plated per well.

Results

Abnormal testicular development in adult male chimeric animals

Chimeric mice that are determined to be at least 75% derived from *Wt1*−/− cells by coat color (adults) or GPI analysis (embryos) are called "highly chimeric." Highly chimeric adult animals were examined for developmental abnormalities. Among the abnormalities observed were unilateral renal agenesis, unilateral or bilateral renal hypoplasia (small but histologically normal kidneys), malformed spleens, uterine and oviductal abnormalities, and dysgenic ovaries (small or misshapen ovaries with normal histology). This paper will focus on the testicular malformations observed in these animals. No difference was observed between the two ES cell lines analyzed; therefore, the results from both were pooled. Thirty percent of the highly chimeric males were cryptorchid and 39% showed hypoplastic testes (unilateral or bilateral; Table 1). Hypoplastic testes were smaller than normal and contained patches of seminiferous tubules devoid of spermatogonia characterized as Sertoli cell-only tubules (Fig. 1). These were often present in the testes of chimeric males, even those with correctly descended testes of normal size. In severe cases, spermatogonia were absent from all seminiferous tubules of a testis. More often, dysgenic tubules containing few or no spermatocytes were found in patches, with histologically normal areas interspersed with abnormal areas.

To determine if the chimeric males were fertile, 10 highly chimeric males were mated with wild-type C57BL/6 females. Five of the 10 chimeric males were fertile. Of these, two produced litters that contained *Wt1*+/− pups, indicating that the *Wt1*-null germ cells can produce functional spermatids.

Table 1
Phenotypic analysis of chimeric animals[a]

Gonad[b]	No. of individuals	Hypoplastic gonads		Cryptorchidism	
		Unilateral	Bilateral	Unilateral	Bilateral
Male	23	4	5	2	5
Female	17	3	9	N/A	N/A
None	1	N/A	N/A	N/A	N/A

N/A: Not applicable.
[a] All animals had >80% ES cell contribution by coat color.
[b] Determined by morphological and/or histological analysis of the gonad.

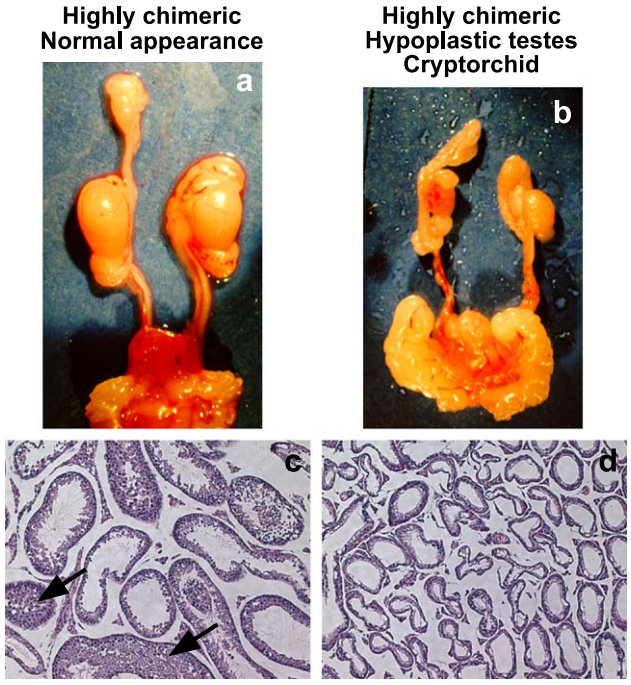

Fig. 1. Phenotype of adult male chimeras. Two representative examples of highly chimeric male reproductive tracts are shown (a and b). (a) A chimeric male with no apparent defects in development of the reproductive tract. (b) A cryptorchid male with hypoplastic testes. (c and d) Histological analysis of the testes from animals shown in panels a and b, respectively. All show some level of impaired spermatogenesis, although areas of normal spermatogenesis (indicated by arrows) are seen in panel c. Many Sertoli cell-only cords can be seen in panel d.

Wt1 function is required to masculinize XX host embryos

Wild-type XY cells are known to masculinize XX host embryos at high efficiency by inducing testicular instead of ovarian development in the indifferent gonad of XY<–>XX chimeric embryos (Patek et al., 1991). In contrast, the ratio of adult males to females obtained after injection of *Wt1*-null XY ES cells did not vary significantly from a 1:1 ratio ($P > 0.1$), suggesting that the phenotype of the chimeric animals is largely determined by the sex chromosome constitution of the host blastocyst (Table 1). In XY<–>XX animals, gonads often transiently develop as ovotestes and then resolve into either ovaries or testes (Bradbury, 1987; Nagamine et al., 1998). To determine if injection of *Wt1*−/− ES cells leads to the development of ovotestes, chimeric E13.5 embryos were examined for the presence of male-specific gene expression (Fig. 2). Detection of *XIST* gene expression by RT-PCR was used to identify the sex of the host embryo (data not shown). *XIST* is only expressed in XX cells at this developmental stage, so only XY<–>XX chimeras will be positive for *XIST* expression. GPI isozyme analysis was used to determine relative chimerism; this assay can

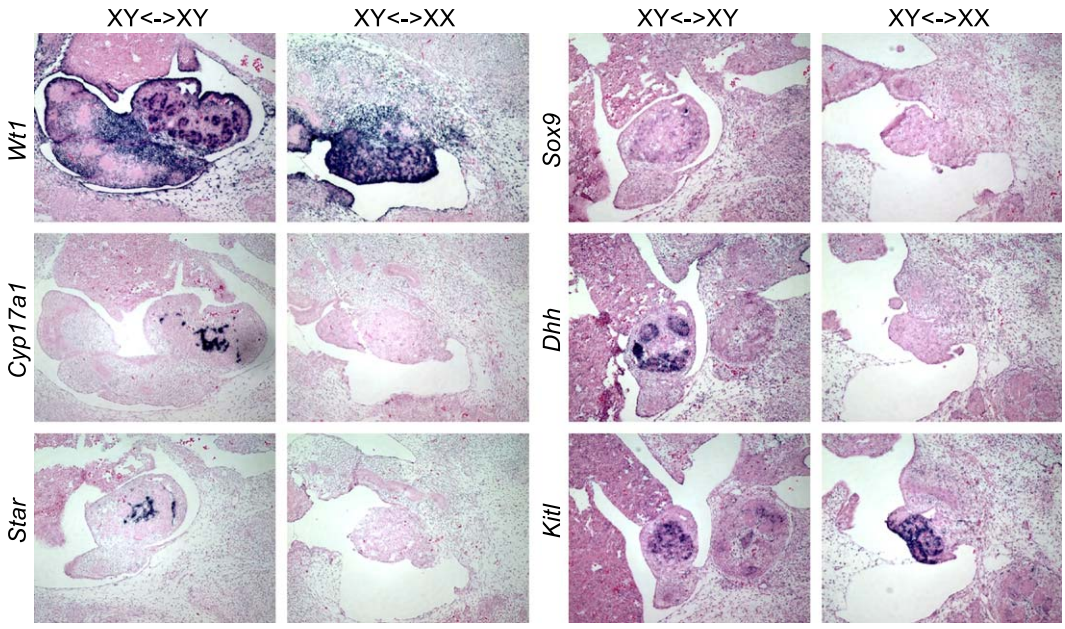

Fig. 2. Analysis of male-specific gene expression patterns in chimeric E13.5 embryos. E13.5 chimeric embryos resulting from the injection of XY *Wt1*-null ES cells into XY ("XY<–>XY" columns) or XX ("XY<–>XX" columns) host blastocysts were used for in situ hybridization to detect *Wt1*, *Sox9*, *Cyp17a1*, *Dhh*, *Star*, or *Kitl* expression (indicated to the left of each pair). No male-specific gene expression is detected in blastocysts derived from a female host embryo. Additionally, *Wt1* and *Kitl* are localized to the developing sex cords in the gonads of male embryos at this stage, while expression in the female gonad is more diffuse. Highly chimeric females demonstrate the female expression pattern for *Wt1* and *Kitl*. Results shown are representative of 6 $XY^{Wt1-/-}$<–>XY and 6 $XY^{Wt1-/-}$<–>XX animals examined.

quantitatively distinguish the distinct isoforms of glucose phosphate isomerase present in the ES cells and host cells. Animals with a high *Wt1*-null XY cell contribution often showed reduced *XIST* expression relative to those with lower ES cell contributions as determined by GPI isozyme analysis (data not shown). Six $XY^{Wt1-/-}$<–>XY and six $XY^{Wt1-/-}$<–>XX chimeric E13.5 embryos were examined, all with 50–90% ES cell contribution (determined by GPI isozyme analysis). Four male-specific markers were analyzed: the Sertoli cell markers *Sox9* and *Dhh* and the Leydig cell markers *Cyp17a1* and *Star*, all of which are expressed in the gonads of XY but not XX animals at E13.5 (Fig. 2 and data not shown). Additionally, *Wt1* expression and Kit ligand (*Kitl*, also known as stem cell factor) expression were analyzed; these genes are expressed in both male and female embryos but show distinct expression patterns. In males, they normally become localized to the developing sex cords; while in females, they usually demonstrate a more diffuse staining pattern. No expression of any male-specific markers was found in $XY^{Wt1-/-}$<–>XX chimeras, and the pattern of *Wt1* and *Kitl* expression was consistent with the female expression pattern (Fig. 2). Thus, unlike wild-type XY ES cell lines, the *Wt1*-null cells are unable to induce male-specific gene expression in an XX embryo, even in high percentage chimeras. Additionally, no histological evidence of ovotestes formation was observed. Therefore, the transient development of ovotestes in these chimeras occurs infrequently if at all.

All somatic lineages of the adult testes are present in chimeric animals

To determine if the lack of spermatogonia in $XY^{Wt1-/-}$<–>XY chimeric males is the result of loss of a specific somatic testicular cell type, we attempted to determine if any somatic cell lineages are absent or underrepresented in these animals (Fig. 3). Because *Wt1* is highly expressed in Sertoli cells, we first tested the hypothesis that a deficiency of Sertoli cells was responsible for the dysgenic seminiferous tubules. *Wt1* was used as a marker for Sertoli cells; this has the additional advantage of only labeling the host-derived Sertoli cells. *Wt1*-positive cells were observed lining the base of severely dysgenic seminiferous tubules using in situ hybridization (not shown) or immunohistochemistry (Fig. 3). Additionally, expression of the Sertoli cell markers *Sox9*, *Dhh*, and *Kitl* was detected in severely dysgenic testes, demonstrating that dysgenic tubules were lined with functional Sertoli cells derived from the wild-type host cells (not shown). To determine if Leydig cells were underrepresented or nonfunctional in these animals, expression of the Leydig cell markers *Star*, *Cyp17a1*, and *Lhcgr* was examined (Fig. 3 and data not shown). Leydig cells were observed in all testes examined. To verify that the peritubular myoid cells were present, smooth muscle actin immunostaining was employed (Fig. 3). All seminiferous tubules were lined by smooth muscle actin-positive cells. Therefore, all of the somatic cell lineages were present within dysgenic testes, and the absence of a specific somatic

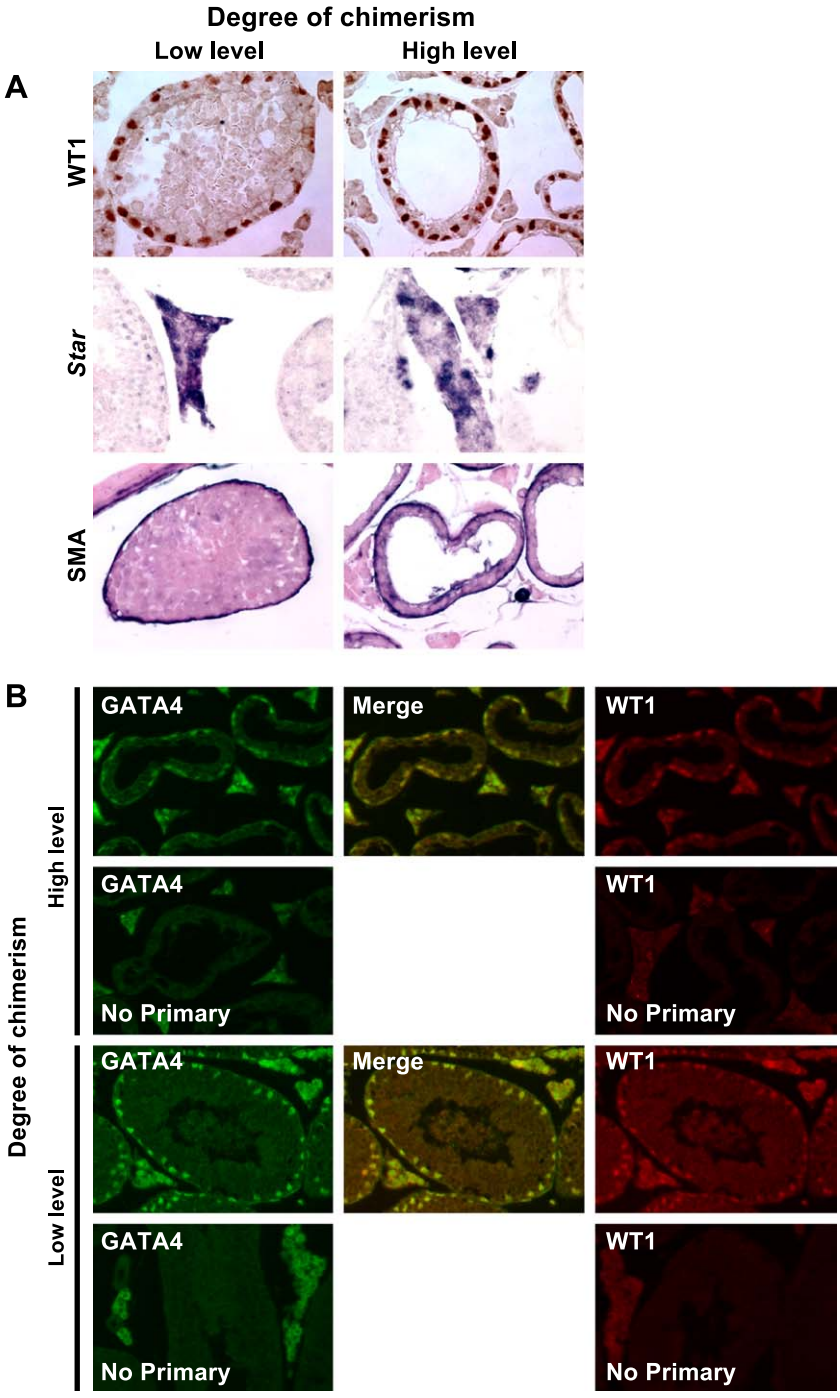

Fig. 3. Analysis of the somatic cell composition of adult chimeric testes. (A) Testes from animals with a low (left panels) or high (right panels) level of chimerism were analyzed for the expression of WT1 (top panels; immunohistochemical stain, brown nuclear staining in Sertoli cells), *Star* (middle panels; in situ hybridization, blue cytoplasmic staining in Leydig cells), or smooth muscle actin (bottom panels; immunohistochemical stain, blue cytoplasmic signal in peritubular myoid cells and vascular smooth muscle cells). Testes showing severe defects in spermatogenesis do not show loss of any somatic cell lineage. (B) All Sertoli cells in Sertoli cell-only cords express WT1. High- or low-level chimeric animals were examined for the presence of WT1-negative Sertoli cells using immunofluorescent labeling with anti-GATA4 (green) and anti-WT1 (red) antibodies. No GATA4-positive, WT1-negative Sertoli cells were observed.

lineage cannot account for the dysgenic appearance of the seminiferous tubules. However, these results do not eliminate the possibility that one or more somatic lineages exhibit a functional deficiency despite the normal pattern of gene expression.

The data described above indicate that Sertoli cells are present in these animals; however, it was possible that a subpopulation of *Wt1*-negative Sertoli cells contributed to the loss of germ cells. To test this possibility, immunofluorescent labeling was used to examine WT1 expression in

the total Sertoli cell population, which was identified using an antibody to GATA4 (Fig. 3B). All GATA4-positive also expressed WT1, suggesting that all Sertoli cells were derived from wild-type host cells.

Chimeric embryos show impaired germ cell population of the gonad

The presence of all somatic cell lineages in dysgenic testes suggested that the reduction of spermatogonia might be due to impaired germ cell colonization of the embryonic gonad. Therefore, E16.5 chimeric gonads were analyzed for the presence of germ cells by staining for alkaline phosphatase activity, a marker of PGCs (Fig. 4). Laminin immunostaining was used to outline the seminiferous tubules. Control embryos showed strong alkaline phosphatase staining within the seminiferous tubules. In contrast, highly chimeric embryos showed little alkaline phosphatase staining within the seminiferous tubules. Additionally, some tubules showed no evidence of alkaline phosphatase-positive germ cells. Therefore, the germ cell population of the embryonic gonad is deficient in chimeric animals, suggesting a role for *Wt1* in germ cell migration or survival in the developing gonad.

Wt1 is expressed in germ cells

PGCs are present in the urogenital ridge of *Wt1*−/− embryos at E11.5 (Kreidberg et al., 1993), indicating that *Wt1* is not required for PGC survival before E11.5 or for migration to the urogenital ridge. Thus, a reduced germ cell population in the gonads of E16.5 chimeric mice suggests that *Wt1* is involved in germ cell survival or migration after E11.5, that is, beginning at the time when germ cells migrate into the emerging gonad. As a first step to address this issue, *Wt1*-null ES cells were tagged with a GFP expression vector to assess the ability of *Wt1*-null cells to contribute to the different cell lineages within the gonad. However, in high percentage chimeras, GFP-expressing cells contributed poorly to the chimeric gonad (data not shown). Thus, this experiment was uninformative about the requirement for *Wt1* in specific lineages in the gonad, except to indicate that *Wt1*-null cells did not contribute to the germ cell population in a robust fashion. These observations, combined with the previous results indicating a normal complement of Sertoli and Leydig cells in chimeric testes, suggested that *Wt1* may indeed be important in developing germ cells. Therefore, we examined *Wt1* expression in germ cells.

The widespread expression of *Wt1* in the early embryonic gonad makes it difficult to distinguish expression in distinct lineages (for example, see Fig. 5). To determine if *Wt1* is expressed in individual germ cells, isolated gonads from wild-type E11.5, 12.5, and 13.5 embryos were disaggregated, and immunofluorescent labeling was used to analyze WT1 expression in germ cells (Fig. 5). SSEA-1 immunostaining was used to identify germ cells. Disaggregation was necessary because SSEA-1 is a cell surface epitope, and it would otherwise be difficult to ascertain that the two signals arise from the same cell and not adjacent cells. The percentage of SSEA-1-positive germ cells expressing WT1 increased from E11.5 to E13.5; by E13.5, all SSEA-1-positive cells also expressed WT1 (Table 2). To confirm this result, RNA was isolated from a pure population of germ cells isolated by FACS at E10.5 and E12.5 and subjected to RT-PCR analysis for *Wt1* (Fig. 5D). *Wt1* expression was detected at E12.5 in both purified germ cell RNA samples and total gonadal RNA samples but was not detected in RNA samples from E10 germ cells. *Renin* (a marker of somatic lineages; Perera et al., 2001) was detected in gonadal RNA samples, but not germ cell RNA samples at either E10.5 or E12.5. *Oct3/4* (a germ cell marker) was detected in all samples. Therefore, the combined data suggest that *Wt1* expression is activated in germ cells around E11.5. This time point is shortly after the germ cells have entered the gonad in wild-type animals and corresponds to the transition from migratory germ cells to gonadal germ cells. No difference in germ cell expression of *Wt1* was seen between male and female embryos. Therefore, *Wt1* is expressed in the germ cells of both sexes during gonadal development.

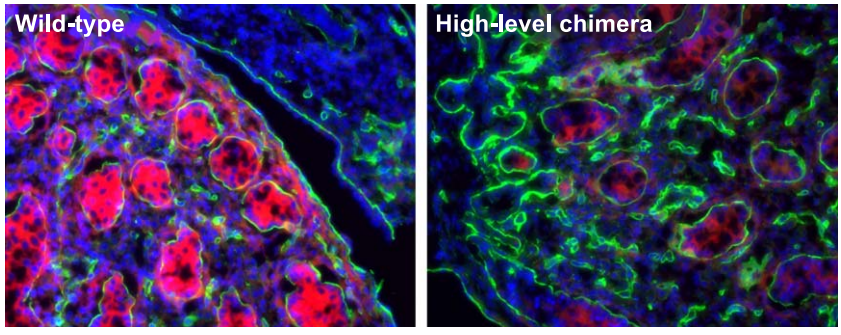

Fig. 4. Analysis of germ cell contribution to chimeric testes. Testes from E16.5 control (left) or highly chimeric (right) embryos were costained for alkaline phosphatase activity (red signal) and laminin (green signal). Nuclei were counterstained with DAPI (blue signal). Testes from a highly chimeric animal show greatly reduced alkaline phosphatase activity.

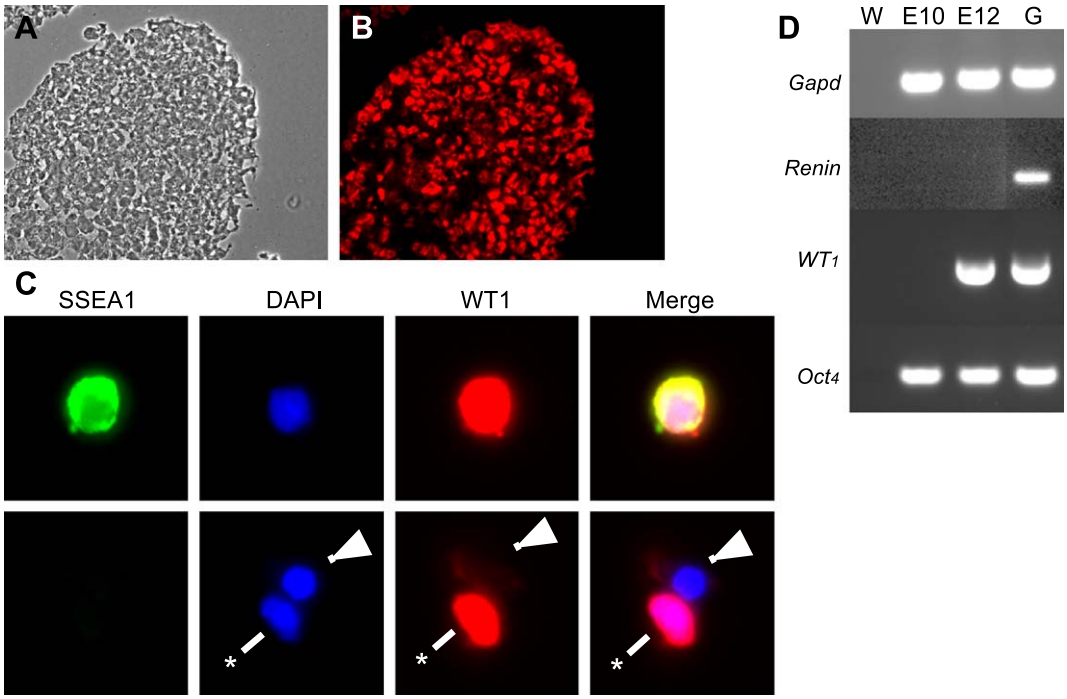

Fig. 5. *Wt1* expression in embryonic germ cells. (A) Phase contrast image of a wild-type E11.5 gonadal ridge. (B) Immunofluorescent detection of WT1 (red) in the gonadal ridge shown in panel (A). Note the widespread expression of WT1 in the gonad at this stage. (C) Expression of WT1 in disaggregated gonadal cells at E12.5. Disaggregated cells were costained for WT1 (red) and SSEA-1 (green) and counterstained with DAPI (blue). SSEA-1 is a germ cell marker. A WT1-positive, SSEA-1-positive cell is shown at the top. An SSEA-1-negative, WT1-positive cell (*) and an SSEA-1-negative, WT1-negative cell (arrowhead) are shown at the bottom. (D) RT-PCR analysis of *Wt1* expression in purified primordial germ cells. PCR was performed on cDNAs from purified primordial germ cells at E10 (E10) or E12 (E12). As a positive control, E12 total gonadal cDNA was used as a template (G); water was used as a negative control (W). Primer pairs tested are as follows: (Gapd), *Gapd*, as a control for cDNA synthesis; (Renin), *Renin*, as a negative control for somatic cell contamination; (WT1), *Wt1*; (Oct4), *Oct3/4*, a germ cell marker. No signals are detected in the water control, whereas all primers amplify products from total E12 gonadal cDNA. *Wt1* expression is detected in purified germ cells at E12 but not E10; in contrast, no renin is detected in either purified germ cell sample.

Wt1 function is important for germ cell survival

In *Wt1*-null embryos, gonadal development and germ cell migration proceed normally until E11.5. At this point, gonadal development is arrested in mutant embryos (Kreidberg et al., 1993). To examine *Wt1*-null germ cell development beyond this stage, an in vitro germ cell culture system was employed. The gonadal ridge and adjacent mesonephric region from individual E11.5 embryos derived from *Wt1*+/− heterozygous intercrosses were disaggregated and plated onto an irradiated STO feeder layer. Cells were fixed 90 min or 48 h after plating and alkaline phosphatase-positive cells were counted (Fig. 6). Ninety minutes after plating, a small reduction in the number of germ cells was observed in mutant embryos compared to wild-type animals (Fig. 6A). After 48 h, cultures derived from mutant embryos showed a significant reduction in the number of germ cells. At this point, the AP-positive cells in cultures derived from wild-type animals were clustered and rounded, while the AP-positive cells in mutant cultures tended to be isolated and elongated (not shown). Therefore, *Wt1* is important for germ cell survival and/or proliferation in culture.

To rule out the possibility that the loss of *Wt1*−/− germ cells in culture was secondary to the loss of somatic cells, we devised a coculture system to examine the survival of *Wt1*−/− germ cells in the presence of wild-type somatic cells (Fig. 6B). Wild-type gonads were depleted of germ cells by busulfan injection on E9.5; E11.5 gonads were then disaggregated to generate the feeder layer. Busulfan treatment was used to reduce the number of wild-type germ cells in the cultures; approximately 80% depletion of wild-type germ cells was achieved (data not shown). Germ cells were isolated from *Wt1*+/+, *Wt1*+/−, and *Wt1*−/− E11.5 embryos by incubation in PBS + EDTA followed by mechanical

Table 2
WT1 expression in SSEA-1-positive cells

WT1	E11.5	E12.5		E13.5	
	Mixed[a]	Male[b]	Female[c]	Male[d]	Female[e]
Negative	8	0	2	0	0
Positive	11	14	23	24	54
Total	19	14	25	24	54
Percent WT1 positive	57.9	100.0	92.0	100.0	100.0

[a] 370 total cells counted.
[b] 152 total cells counted.
[c] 166 total cells counted.
[d] 224 total cells counted.
[e] 207 total cells counted.

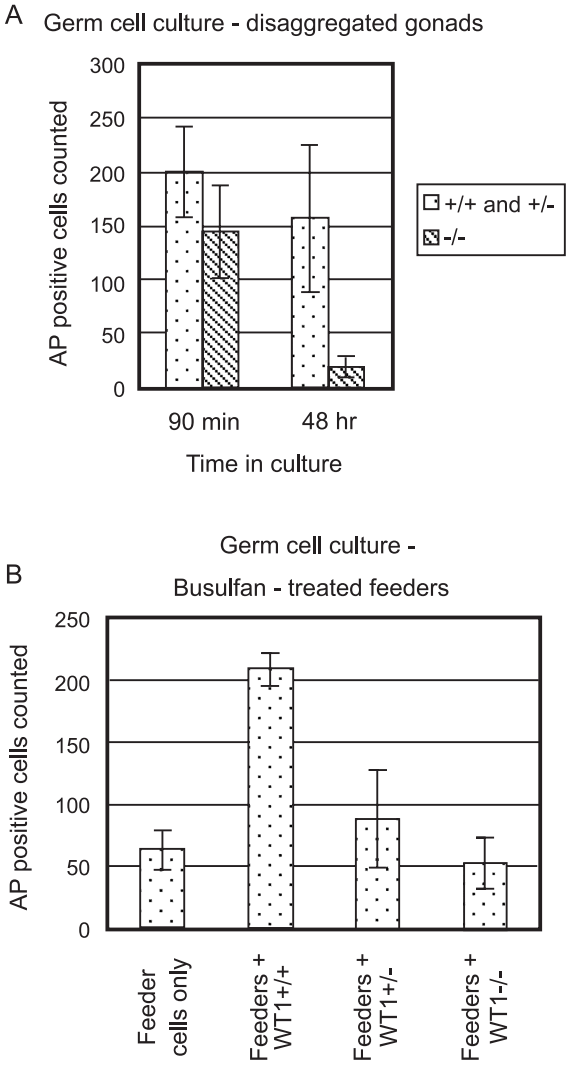

Fig. 6. In vitro culture of germ cells. Gonadal ridges were isolated from *Wt1*+/+, +/−, and −/− embryos, disaggregated and plated onto irradiated STO feeders for 90 min or 48 h. No difference in cultured germ cell numbers was detected between *Wt1*+/+ and +/− animals under these conditions. Therefore, data from +/+ and +/− embryos were combined. (A) Chart showing the mean number of AP-positive cells counted after 90 min (diagonal striped bars) or 48 h (stippled bars). Error bars indicate one standard deviation. (B) Survival of *Wt1*+/+, +/− and −/− germ cells on a somatic cell feeder layer. Somatic cells from E11.5 wild-type gonads were obtained from busulfan-treated animals (to deplete endogenous germ cells) and cultured alone (feeder only) or cocultured with *Wt1*+/+, *Wt1*+/−, or *Wt1*−/− germ cells. Isolated germ cells failed to grow in the absence of feeders. A representative experiment is shown. The mean number of germ cells remaining after 48 h is shown; error bars indicate one standard deviation.

separation. Isolated germ cells were cocultured with busulfan-treated feeders for 48 h, and AP-positive germ cells were counted. Very few isolated germ cells grew in the absence of feeder cells (generally none; occasionally up to 20; data not shown). As expected, some wild-type germ cells remained in the somatic cell feeder layers (Fig. 6B). When cocultured on a somatic cell feeder layer, wild-type germ cells survived for 48 h (Fig. 6B). In contrast, *Wt1*−/− germ cells survived poorly on a wild-type feeder layer (Fig. 6B). These data demonstrate that *Wt1*−/− germ cells are inefficiently rescued by wild-type somatic cells, further suggesting a role for *Wt1* in germ cell survival.

Discussion

The data presented here demonstrate that *Wt1* functions in the development of germ cells. *Wt1* expression is detected in embryonic germs cells initiating between embryonic day 10.5 and 12.5. Germ cells derived from *Wt1*-null embryos showed impaired growth in culture. Further, *Wt1*-null germ cells are inefficiently rescued by wild-type somatic cells both in vivo and in vitro.

Wt1 is required for development of all gonadal lineages

Mutations in the *Wt1* gene result in urogenital anomalies in both humans and mice (reviewed by Scharnhorst et al., 2001). However, it was not known if the mutations result from a direct requirement for *Wt1* in a limited set of gonadal cells or from a more widespread requirement in all gonadal lineages. The results presented here suggest that *Wt1* mutations can impair the development or function of all gonadal cell types.

The first sign of male sex determination in mammals is when the presumptive Sertoli cell precursors begin to express *Sry* (Koopman et al., 1990). *Wt1* has been suggested to regulate *Sry* expression in vitro and in vivo (Hammes et al., 2001; Hossain and Saunders, 2001). *Sry* expression has been correlated with increased cellular proliferation in the gonad and recruitment of mesonephric cells to form the sex cords, two key events in testicular development (Capel et al., 1999; Schmahl et al., 2000). Additionally, the fetal Sertoli cells produce mullerian inhibiting substance (MIS), which induces mullerian duct regression in males. MIS has been proposed to be a transcriptional target of *Wt1* (Nachtigal et al., 1998). Fetal Sertoli cells also produce *Dhh*, which is critical for development of the fetal Leydig cells (Yao et al., 2002). Many of the urogenital anomalies seen in individuals with mutations in the *Wt1* gene or in transgenic mice lacking specific isoforms of *Wt1* could be explained by impaired Sertoli cell differentiation. *Wt1*-null cells are excluded from the Sertoli cell lineage, providing direct evidence that *Wt1* is required cell autonomously for Sertoli cell proliferation or survival. Therefore, it is not surprising that *Wt1*-null XY cells would fail to masculinize an XX host embryo. However, given the widespread expression of *Wt1* during early gonadal development, it was important to assess the effect of *Wt1* mutations on the other gonadal lineages.

Clinical syndromes resulting from mutations in the *Wt1* gene often result in cryptorchidism and abnormal development of the external genitalia, such as hypospadias, micropenis, or pseudohermaphroditism, suggesting impaired fetal Leydig cell development or function (Grumbach and Conte,

1998; Little and Wells, 1997). The cryptorchidism observed in chimeric animals, similar to humans with *Wt1* mutations, suggests a role for *Wt1* in fetal Leydig cell development or function. Several potential mechanisms could account for this phenotype. First, fetal Leydig cells or a precursor to fetal Leydig cells could require *Wt1* function for development or survival. Although the origin of the fetal Leydig cell lineage has not been firmly established, they are most likely derived from the intermediate mesoderm, which is known to express *Wt1*. Therefore, the possibility that some of the clinically relevant consequences of *Wt1* mutations may reflect a role for *Wt1* in Leydig cell development must be considered. Alternatively, an impairment of Leydig cell function could be secondary to impaired Sertoli cell function, although the presence of WT1 and GATA4-expressing Sertoli cells makes this possibility less likely. Further experimentation will be necessary to distinguish these possibilities.

Wt1 in germ cell development

Unexpectedly, *Wt1*-null ES cells were largely excluded from the germ cell lineage of the developing gonad at embryonic day 16.5. This was surprising because *Wt1* expression had not been reported in germ cells at any stage, and *Wt1*-null embryos show normal germ cell development and migration into the area of the gonadal ridge at embryonic day 11.5 (Kreidberg et al., 1993). Additionally, two of five highly chimeric males transmitted the *Wt1* mutant allele, indicating that the requirement for *Wt1* in germ cells is not absolute. However, the detection of *Wt1* expression in embryonic germ cells using two independent lines of experimentation, combined with the results of the chimeric and in vitro culture studies, indicate that *Wt1* is indeed expressed in germ cells. Together, these results suggest a previously unrecognized role for *Wt1* in germ cell proliferation, maturation, or survival.

The expression of *Wt1* in germ cells and the fact that *Wt1* is a nuclear protein implicated in gene regulation suggests that the role of *Wt1* in germ cells is cell autonomous. According to this model, loss of *Wt1* in germ cells would lead to cell-autonomous germ cell lethality. However, the observed survival of some *Wt1*-null germ cells would indicate that the requirement for *Wt1* is not absolute. Alternative models not requiring strict cell-autonomous *Wt1* function could explain these results. For example, *Wt1* may regulate the expression of a secreted or cell-surface protein necessary for germ cell survival. In this case, *Wt1*-null germ cells would be lost if they are entirely surrounded by other *Wt1*-null germ cells, as might occur in highly chimeric embryos. A small number of *Wt1*-null germ cells in contact with wild-type germ cells would be exposed to this hypothetical survival factor, thereby bypassing the internal requirement for *Wt1*. The mechanism of *Wt1* action in germ cells remains to be determined.

The expression of *Wt1* in germ cells appears to begin as they convert from primordial germ cells to gonadal germ cells (Donovan et al., 1986; Enders and May, 1994; Tanaka et al., 2000). At E11.5, only a slight reduction in the number of germ cells is detected in the gonads of *Wt1*-null embryos. However, after 48 h in culture, *Wt1*-null germ cells are lost, suggesting that *Wt1* is important for gonadal germ cell survival or proliferation. *Wt1*-null germ cells are inefficiently rescued by wild-type somatic cells, as suggested by data from both in vitro recombination experiments and chimeric animals. Therefore, despite the survival of some *Wt1*-null germ cells, the data suggest a primary role for *Wt1* in germ cells. Thus, the onset of *Wt1*, GCNA, and *Mvh* expression provides a molecular basis for distinguishing gonadal germ cells from primordial germ cells (Donovan et al., 1986; Enders and May, 1994; Toyooka et al., 2000). The coexpression of *Wt1* in gonadal germ cells and Sertoli cells is intriguing. It occurs at a time when gonadal germ cells and Sertoli cells must begin to intimately associate with each other in seminiferous tubules. One hypothesis is that *Wt1* stimulates the expression of a homophilic adhesion molecule expressed on both gonadal germ cells and Sertoli cells, which mediates their interaction. Since germ cell specification is independent of *Wt1* function, but gonadal germ cell survival or proliferation requires *Wt1*, the number of spermatogonia in chimeric animals may be limited by the proportion of wild-type primordial germ cells that initially populate the seminiferous tubules.

Because wild-type cells were apparently able to compensate for the inability of *Wt1*-null cells to populate the gonad, the question may be raised as to why wild-type germ cells did not similarly compensate for the *Wt1*-null cells and provide a normal complementation of germ cells to the developing or adult gonad. It is known that germ cell proliferation is limited to a very few, probably no more than three, rounds of cell division before entering a period of quiescence (De Rooij and Dissel-Emiliani, 1997). Given that PGCs do not require *Wt1* before E11.5 (Kreidberg et al., 1993), it would be expected that the population of PGCs in the highly chimeric animals would be composed mainly of *Wt1*-null cells. Thus, once in the gonad, the limited number of germ cell divisions would severely limit the ability of a small population of wild-type germ cells to fully populate the gonad. Therefore, the observation that wild-type germ cells could not compensate for the loss of *Wt1*-null germ cells to provide a normal complement of germ cells in the developing testes is not surprising. Moreover, our results suggest that if there is deficient population by germ cells of some testicular cords during embryonic development, there may not be adequate compensatory proliferation of spermatogonia during later development or adult life to provide a normal population of spermatogonia in mature seminiferous tubules.

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