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Sox3 expression in undifferentiated spermatogonia is required for the progression of spermatogenesis

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Abstract

Sox3, a member of the high mobility group (HMG) family of transcription factors, is expressed in neural progenitor cells and in the gonads. Targeted deletion of *Sox3* in mice causes abnormal development of the diencephalon and Rathke's pouch, the progenitor of the anterior pituitary gland. Male and female mice are also infertile and exhibit a primary defect in gametogenesis. In this study, we examined the expression and function of Sox3 in C57BL/6 mice to better understand its role in spermatogenesis. Testis development was normal during embryogenesis. However, spermatogenesis failed to progress during the postnatal period, with germ cell loss beginning at postnatal day 10 (P10). By P14, *Sox3* null mice were nearly agametic, retaining only Sertoli cells and undifferentiated spermatogonia. Pituitary gonadotropin and testosterone levels were normal, suggesting a defect in Sertoli cell and/or germ cell function. Immunostaining revealed that Sox3 was expressed in a subpopulation of germ cells localized at the base of the seminiferous tubules. Sox3 expression was restricted to proliferating germ cells and colocalized with neurogenin 3 (Ngn3), a helix–loop–helix transcription factor implicated in spermatogonial differentiation. The absence of Sox3 decreased Ngn3 and increased expression of Oct4, a marker of undifferentiated spermatogonia. We conclude that Sox3 is expressed in A_s, A_{pr} and A_{al} spermatogonia and is required for spermatogenesis through a pathway that involves Ngn3.

Keywords: Sox3; Neurogenin3; Oct4; Stem cells; Germ cells; Spermatogonia; Spermatogenesis

Introduction

The Sox (Sry-related HMG box) family of transcription factors is defined by homology to the DNA-binding domain (high mobility group; HMG) of the male-determining factor Sry (Collignon et al., 1996; Gubbay et al., 1990). There are twenty Sox genes in mice and humans (Bowles et al., 2000; Schepers et al., 2002). *Sox3* is a single-exon gene located on the X chromosome and is thought to be the ancestral precursor from which the Sry gene evolved (Collignon et al., 1996; Foster and Graves, 1994). Phylogenetic studies place *Sox3* in the B1 group of the Sox family, which also includes *Sox1* and *Sox2*.

Group B1 Sox genes are expressed by progenitor cells throughout the developing central nervous system (CNS) (Collignon et al., 1996; Brunelli et al., 2003; Uchikawa et al., 1999; Wood and Episkopou, 1999). Sox genes are generally downregulated by neural cells as they exit the cell cycle and begin to differentiate (Bylund et al., 2003; Kan et al., 2004; Pevny et al., 1998; Uchikawa et al., 1999), a pattern consistent with a regulatory role for these proteins in the initiation or progression of neuronal differentiation (Bylund et al., 2003; Kan et al., 2004; Pevny et al., 1998; Uchikawa et al., 1999). *Sox3* mRNA is also expressed in the developing urogenital ridge (Collignon et al., 1996) and later in the adult gonad (Shen and Ingraham, 2002) of both sexes.

Sox3 and Sry have overlapping DNA binding specificity (Bergstrom et al., 2000). This feature, combined with the evolutionary relationship of these proteins, led to the hypothesis that Sox3 might be involved in sex determination (Graves, 1998). However, deletion of *Sox3* in 129 Sv/Ev mice did not induce sex reversal (Rizzoti et al., 2004;

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Weiss et al., 2003), though defects in hypothalamic/pituitary development and spermatogenesis were observed.

Within the testis, the exact site of *Sox3* expression is uncertain. RNAse protection assays (Collignon et al., 1996) and in situ hybridization (Shen and Ingraham, 2002) suggested that *Sox3* was expressed in somatic cells of the embryonic gonad and in Sertoli cells of the adult testis. On the other hand, *Sox3* has been detected in the primordial germ cells of avian embryos (Uchikawa et al., 1999) and in a subset of spermatogonia in mice (Rizzoti et al., 2004). The latter observation is potentially informative. In addition to more differentiated cell types, the spermatogonia include the male germline stem cells, which are analogous to the Sox-positive neural progenitor cells noted above.

In male mice, germ cell competence is induced at embryonic day (E) 6.5 in proximal epiblast cells by signals emanating from the extraembryonic ectoderm (Lawson et al., 1999). During the specification period (E7.5), these precursor cells give rise to primordial germ cells (PGC) that migrate through the dorsal mesentery and reach the genital ridges between E10.5 and E12.5, where they are enclosed by somatic cells and become gonocytes (Ginsburg et al., 1990). The gonocytes proliferate for several days and then arrest in G0/G1 phase until after birth, when they resume proliferation and initiate spermatogenesis. By postnatal day (P) 2–3, the majority of gonocytes resume mitosis and migrate to the basement membrane of the seminiferous tubule (McLean et al., 2003). By P4, gonocytes that reach the basement membrane begin to differentiate into spermatogonial stem cells (A_{single} or A_s) that are competent to initiate the first wave of spermatogenesis.

Progeny of A_s spermatogonia renew the stem cell population or they can remain connected by intercellular bridges to form pairs (A_{paired} or A_{pr}) that undergo additional divisions to form groups of four, eight or more cells ($A_{aligned}$ or A_{al}) (reviewed in de Rooij and Grootegoed, 1998). A_s, A_{pr} and A_{al} are morphologically undifferentiated spermatogonia, and they proliferate independent of more mature cells (A_1 through A_4 and B spermatogonia), which undergo a regimented series of mitotic divisions characteristic of the epithelial cycle of spermatogenesis (de Rooij, 2001; McLaren, 2000; Meistrich, 1993). Type B spermatogonia give rise to primary spermatocytes, which undergo two meiotic divisions to yield the haploid spermatids that mature to form the spermatozoa (de Rooij and Russell, 2000).

In the current study, we have further characterized the population of cells that express Sox3 and provide evidence that Sox3 plays a role in spermatogonial differentiation.

Materials and methods

Sox3 null mice

Generation of *Sox3* null mice on the 129Sv/j background strain has been described previously (Weiss et al., 2003). For the current experiments, $Sox3^{-/+}$ females were bred to WT C57BL/6 males (Jackson Labs, Bar Harbor, ME), and mice from the F5 and F6 generations were studied. All animals were housed in a barrier facility under normal light and dark conditions with free access to food and water. All procedures were approved by the Northwestern University Animal Care and Use Committee, and all experiments were performed in accordance with the NIH *Guide for the Care and Use of Laboratory Animals*.

Mice were genotyped for the presence or absence of *Sox3* and *Sry* using genomic DNA from tail snips as described previously (Weiss et al., 2003). For studies from birth through adulthood, WT and B6-$Sox3^{-/Y}$ males were sacrificed by cervical dislocation after anesthesia, and the testis were quickly dissected. For studies of embryonic gonads, breeders were placed in the same cage at 16:00 h, and females were examined for the presence of a vaginal plug before 08:00 h the following morning. For successful matings, the first morning was designated as 0.5 dpc, and females were euthanized, and embryonic gonads were dissected on days 11.5, 13.5 and 14.5 dpc with the aid of an MZLF III dissecting microscope (Leica, Heerbrugg, Switzerland). Genotyping was performed using genomic DNA prepared from unused segments of individual embryos.

Sperm counts and motility

Eight-week-old males were killed by cervical dislocation after anesthesia, and the caudae epididymides were quickly dissected and placed into 1 ml activated (2% BSA) Quinn's HTF medium (SAGE BioPharma, Bedminster, NJ). Each epididymis was diced, and the sperm were allowed to disperse into the medium for 10 min at 37°C. Aliquots (20 μl) of a 1:10 dilution of the epididymal suspension were counted on a hemacytometer. Sperm that showed tail motion were designated as motile.

Histology and immunohistochemistry

Testes were fixed in 10% phosphate-buffered formalin and embedded in paraffin. All sections were cut at a thickness of 4 μm, and images were taken using a Zeiss Axioskop microscope (Thornwood, NY) and an Optronics MicroFire digital camera (Goleta, CA). Hematoxylin and eosin staining was performed using standard protocols. For immunochemistry, sections were deparaffinized in xylene and rehydrated in descending concentrations of ethanol followed by antigen retrieval in sodium citrate buffer (microwave 10 min at full power). Sections were blocked in 10% normal serum for 30 min and incubated with primary antibody at 4°C overnight, with the exception of Gcna1, which was incubated for 1 h at 33°C. Primary antibodies were rabbit anti-laminin (1:200, Sigma-Aldrich), goat anti-Gata4 (1:200), goat anti-MIS

(1:200), goat anti-Ngn3 (1:100), mouse anti-Pcna1 (1:200), mouse anti-Oct4 (1:100, all from Santa Cruz Biotechnology), mouse anti-γH2AX (1:100, Upstate Biotechnology), rabbit anti-Sox3 (1:500, provided by T. Edlund, Umea, Sweden), rabbit Anti-3βHSD (1:5000, provided by I. Mason, Edinburgh) and rat monoclonal anti-Gcna1 (provided by George Enders, University of Kansas Medical Center).

Slides were washed with PBS + 0.1% Tween 20 and incubated with Cy3 or FITC conjugated species-specific secondary antibodies for 2 h at room temperature (1:200, Jackson Immunoresearch). After additional washes, sections were incubated with 0.1% Sudan Black in 70% ethanol to block autofluorescence, washed with tap water and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA).

RNA extraction and RT-PCR

Total RNA was isolated from individual testes using Trizol (GIBCO-BRL) according to the manufacturer's protocol. RNA (2 μg) from individual samples was treated with DNAse I (Ambion) and reverse transcribed using AMV RT (Promega) in a total volume of 20 μl then diluted to a final volume of 50 μl. Minus-RT controls were performed in parallel to test for contamination with genomic DNA. PCR was performed in a total volume of 25 μl containing 5 μl of RT as template, 0.1 μl α^{32}P -dCTP (Amersham Biosciences) and *Taq* polymerase and buffers from Promega. PCRs for Sox3, Ngn3, Oct4, C-Ret and GAPDH were carried out for 25–30 cycles of 1 min at 94°C, 1 min at 55°C, 1.5 min at 72°C. PCR for Stra8 was as previously described (Menke et al., 2003). PCR products were separated on 6% polyacrylamide gels. Dried gels were exposed to phosphor screens and quantitated using a Storm-840 phosphorimager (Molecular Dynamics). Primers were 5′-TTCGCACTCGCAGCGCGCGTGCCTCGG-3′ and 5′-ACGGCCAAACTTTCGGTCCTC-3′ for Sox3; 5′-GCTATCCACTGCTGCTTGA-3′ and 5′-CCGGGAAAAGGTTGTTGTGT-3′ for Ngn3 (Yoshida et al., 2004); 5′-CAGCGGTGTCTCCATCCAGTA-3′ and 5′-CCCCTCGCTCGTGTCCTCCAA-3′ for c-Ret; 5′-TGGAGAAGGTGGAACCAACTCCC-3′ and 5′-ACACGGTTCTCAATGCTAGTTCGC-3′ for Oct4; 5′-GAGTCAGGAAGAATATGC-3′ and 5′-AGAGACAATAGGAAGTGTC-3′) for Stra8; 5′-CCCTTCATTGACCTCAACTA-3′ and 5′-CCAAAGTTGTCATGGATGAC-3′ for Gapdh.

Statistical analysis

t tests were used for two-way analyses (wildtype versus knockout). To identify differences in RNA expression over time, samples were compared using one-way ANOVA followed by the Least Squares Difference post-hoc test.

Results

Sox3 KO male mice are sterile on the C57BL/6 background strain

On the 129/Svj background (129), *Sox3* KO mice (*Sox3*$^{-/Y}$) exhibit variable hypogonadism and impaired spermatogenesis (Weiss et al., 2003). The *Sox3* null allele was transferred to the C57BL/6 strain (B6), which often exhibits a greater sensitivity to mutations in genes regulating testis development (Albrecht et al., 2003). Sex reversal was not observed in the B6-*Sox3*$^{-/Y}$ mice. Cranial malformations and dental malocclusion, observed in previous studies on the 129 background (Rizzoti et al., 2004; Weiss et al., 2003), were also absent in B6-*Sox3*$^{-/Y}$ mice.

However, B6-*Sox3*$^{-/Y}$ males were severely hypogonadal (Figs. 1A–C), with testis weights measuring 38% of WT littermate controls (Table 1). Total (Table 1) and motile (not shown) epididymal spermatozoa were absent or severely decreased in B6-*Sox3*$^{-/Y}$ males compared to WT controls. To examine fertility, B6-*Sox3*$^{-/Y}$ males (n = 9) were mated with pairs of WT B6 females for 3 months. Vaginal plugs were documented, but no litters were produced.

Gonadal failure in B6-Sox3$^{-/Y}$ *mice*

Consistent and severe histological defects were observed in B6-*Sox3*$^{-/Y}$ mice, though some degree of spermatogenesis was visible in most testes. In less severely affected tubules, spermatocytes and round and elongated spermatids were present, but spermatogenesis was abnormal, and mature sperm were rarely seen (Fig. 1E, asterisk). More severely affected tubules exhibited no spermatogenesis, though spermatogonia (Fig. 1E, white arrowheads) and Sertoli cells (Fig. 1E, black arrowheads) remained. The most severely affected tubules were completely agametic with only Sertoli cells visible (Fig. 1F, arrowheads). Other abnormalities were also observed, including desquamation of immature germ cells, multinucleated giant cells and clusters of Sertoli cells in the tubular lumen. Normal spermatogenesis was never observed.

Pituitary function and steroidogenesis are normal in B6-Sox3$^{-/Y}$ *mice*

Adult body weight did not differ between B6-*Sox3*$^{-/Y}$ and WT littermates (Table 1). Serum LH and FSH levels were also normal in B6-*Sox3*$^{-/Y}$ males, excluding a functional pituitary deficit in these mice (Table 1).

Abnormalities in the hypogonadal testis of B6-*Sox3*$^{-/Y}$ males appear to be restricted to the seminiferous tubules. Staining for 3βHSD was qualitatively similar in testis of adult mice, regardless of *Sox3* status (not shown), suggesting that the steroidogenic Leydig cells were largely unaffected. Consistent with this observation, testosterone levels were within the normal range for WT and B6-*Sox3*$^{-/Y}$

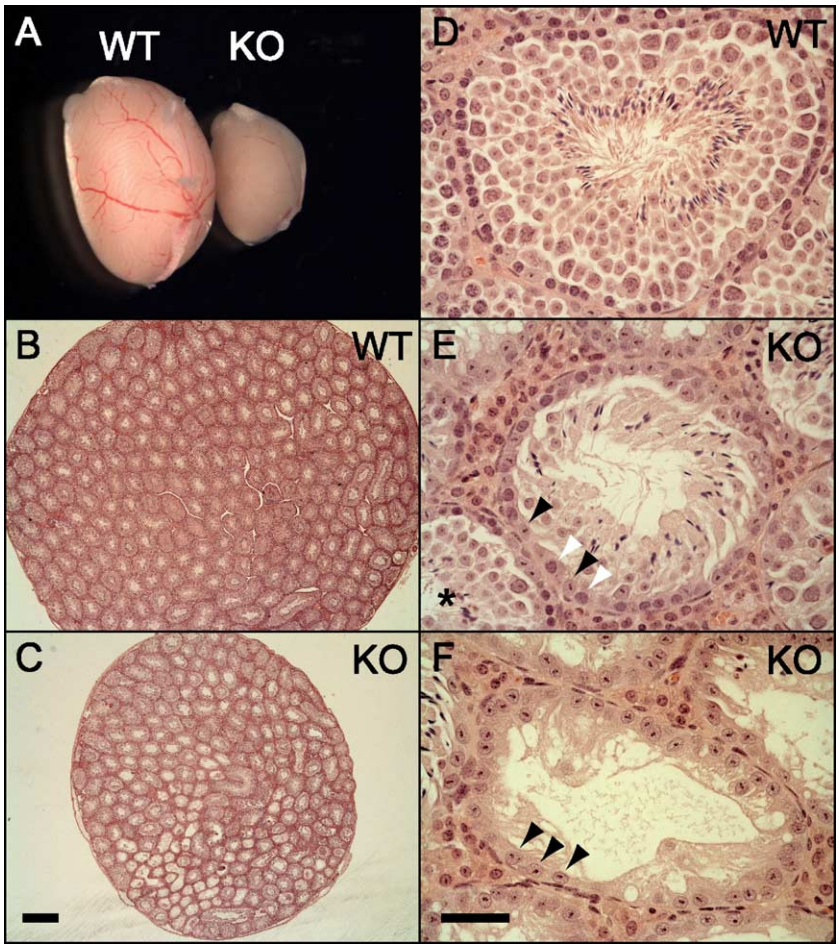

Fig. 1. Testis histology in adult WT and B6-*Sox3*$^{-/Y}$ mice. Whole testis (A) and low power cross-sections (25 × magnification, B and C, scale bar = 500 μm) from 8-week-old WT and B6-*Sox3*$^{-/Y}$ mice. Panels (D) to (F) are higher power sections (400×, scale bar = 50 μm). (D) WT tubule exhibiting normal spermatogenesis. (E) A mildly affected tubule (asterisk) progresses to round spermatids, but mature sperm are not observed. An adjacent tubule is more severely affected, retaining only spermatogonia (white arrowheads) and Sertoli cells (black arrowheads). (F) The most severely affected tubules are Sertoli cells only.

animals (Table 1), and there was no noticeable difference in seminal vesicle size on dissection (data not shown).

Testis degeneration in B6-Sox3$^{-/Y}$ *males is a postnatal defect*

To gain further insight into the timing of testicular degeneration, we examined gonadal development during 11.5–14.5 dpc in the embryo, spanning the critical period of tubule formation, and during P7–P21, which encompasses the initial wave of spermatogenesis.

No structural abnormalities were detected in *Sox3* KO gonads during embryogenesis. H&E staining revealed normal tubule formation in WT and KO animals (Figs. 2Ai and ii). Gata4-positive somatic (Sertoli) cells lined the basal compartment of the tubules. The basement membrane (stained with laminin) was present and formed a contiguous boundary in the developing testis cords (Figs. 2Aiii and iv). The temporal development of each major gonadal cell population was unaffected by the absence of *Sox3*. Sertoli cell expression of Mullerian Inhibiting Substance (MIS), which causes regression of female accessory structures, was similar at 13.5 dpc in tubules from WT and B6-*Sox3*$^{-/Y}$ mice (Figs. 2Bi and ii). Leydig cell populations, monitored by expression of 3βHSD, were also similar in these two groups (Figs. 2Biii and iv), and no differences in germ cell numbers were observed at this time (not shown).

Table 1
Measures of pituitary and testis function (n = 5 per genotype)

	Body weight (g)	Testis weight (mg)	Testosterone (ng/dl)	LH (ng/ml)	FSH (ng/ml)	Total sperm (10^4/ml)
WT	20.3 ± 1.2	83.7 ± 14.4	50.7 ± 51.1	0.33 ± 0.56	41.6 ± 13.7	1370 ± 501.9
KO	20.1 ± 1.6	31.9 ± 4.2	32.2 ± 18.0	0.88 ± 0.57	44.6 ± 19.5	24 ± 53.6
	NS	$P < 0.0001$	NS	NS	NS	$P < 0.001$

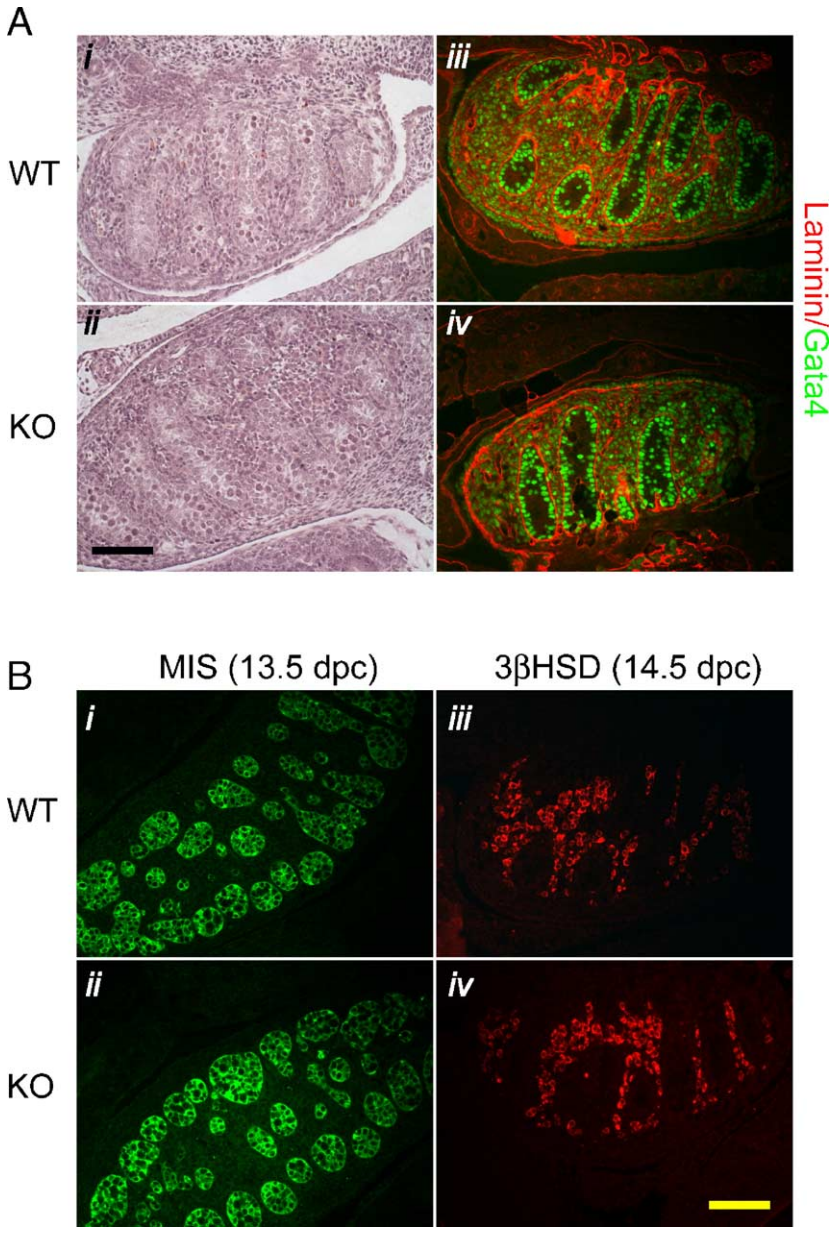

Fig. 2. Gonadal development in WT and B6-*Sox3*$^{-/Y}$ mice. (A) Gonadal structure at 14.5 dpc. H&E staining revealed normal tubule formation in WT (i) and B6-*Sox3*$^{-/Y}$ (ii) testis. Immunostaining with antibodies to laminin (red) and Gata-4 (green) revealed normal structure of the basement membrane and normal organization of the Sertoli cells. Scale bar = 100 μm. (B) Somatic cell populations in the absence of *Sox3*. Immunostaining with antibodies to MIS (13.5 dpc, green, Sertoli cells) and 3βHSD (14.5 dpc, red, Leydig cells) revealed no differences between WT (i, iii) and B6-*Sox3*$^{-/Y}$ (ii, iv) mice. Scale bar = 100 μm.

At 1 week postnatally, no differences were observed in testis weight or histology between WT and B6-*Sox3*$^{-/Y}$ mice (Figs. 3B and C). Testis degeneration was first evident in B6-*Sox3*$^{-/Y}$ mice at P10, during the first wave of spermatogenesis (not shown). By P14, when meiosis would normally have begun, WT testis contained pachytene or secondary spermatocytes, whereas Type A spermatogonia were the only germ cells present in B6-*Sox3*$^{-/Y}$ testis (Fig. 3C, 2 weeks). Testis weights were also significantly lower by 2 weeks of age in B6-*Sox3*$^{-/Y}$ animals (8.5 ± 0.9 mg) compared to control (13.5 ± 1.2 mg) (Fig. 3B, arrow). This difference increased over time, and testis weights in B6-*Sox3*$^{-/Y}$ animals were about 30% of WT from 3 weeks of age through adulthood.

Germ cells fail to differentiate in B6-Sox3$^{-/Y}$ *mice*

Additional staining was performed to explore the loss of differentiated germ cells in B6-*Sox3*$^{-/Y}$ mice. Gcna1 is a germ cell-specific marker expressed in premeiotic cells up to the pachytene stage of meiosis I (Wang and Enders, 1996). Staining of 3-week-old testis with Gcna1 revealed a marked reduction in the total population of germ cells in the absence of *Sox3* (Fig. 4, left panels). To identify the

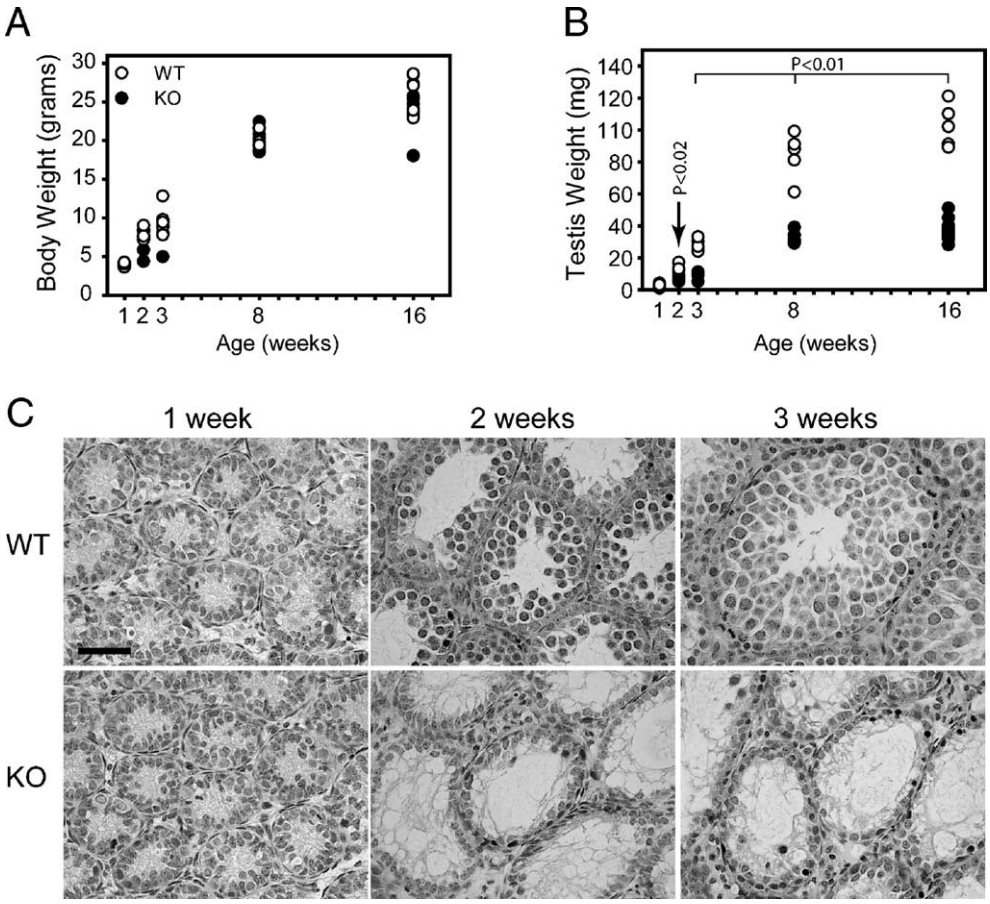

Fig. 3. Postnatal defects in development of B6-*Sox3*$^{-/Y}$ testis. (A) Growth curves. No differences were observed between WT and B6-*Sox3*$^{-/Y}$ males. (B) Testis weights. B6-*Sox3*$^{-/Y}$ males were hypogonadal by 2 weeks of age, and this defect worsened over time. Statistical comparisons refer to WT versus KO at each time point. (C) Histological study of postnatal weeks 1 to 3. No difference was observed at 1 week of age, but severe histological defects were observed in B6-*Sox3*$^{-/Y}$ males by week 2. Note the presence of spermatocytes in WT mice, while only undifferentiated spermatogonia are visible in B6-*Sox3*$^{-/Y}$ mice. By 3 weeks, WT mice have progressed through meiosis, while normal spermatogenesis is absent in B6-*Sox3*$^{-/Y}$, though spermatocytes are occasionally observed. Scale bar = 50 μm.

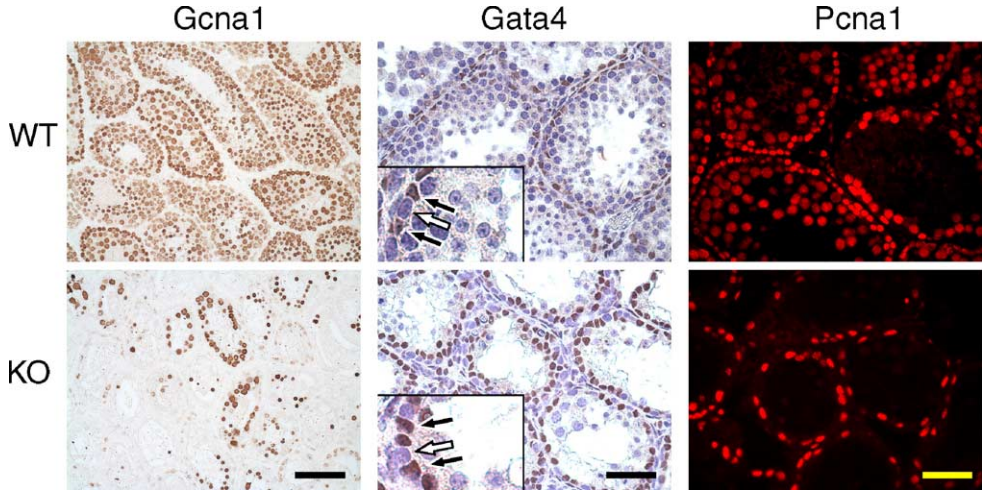

Fig. 4. Germ cell loss in B6-*Sox3*$^{-/Y}$ mice. Immunohistochemistry of 3-week-old testis using an antibody to Gcna1 (brown staining in left panels, scale bar = 100 μm) revealed a vast reduction of germ cell number in the absence of *Sox3*. An antibody to Gata4 (brown staining in center panels, scale bar = 50 μm) marks the Sertoli cells (black arrows) in WT and B6-*Sox3*$^{-/Y}$ mice. Note that the persistence of spermatogonia (white arrows) interspersed with the Sertoli cells and in contact with the basement membrane in B6-*Sox3*$^{-/Y}$ mice. This identification is confirmed by staining of proliferating spermatogonia with an antibody to Pcna1 (red, right panels, scale bar = 50 μm).

remaining cells, tubules were stained with Gata4 (Fig. 4, center panels). Higher magnification (insets) demonstrates that B6-*Sox3*$^{-/Y}$ mice retain Sertoli cells (brown staining [black arrows]) and interspersed round cells, presumably spermatogonia (white arrows). This conclusion is confirmed by staining with Pcna1 (Fig. 4, right panels), a marker of proliferating cells. WT and B6-*Sox3*$^{-/Y}$ mice exhibit a full complement of proliferating spermatogonia in contact with the basal lamina.

To assess whether the absence of differentiated germ cells was due to an increase in apoptosis or a failure of differentiation, TUNEL staining was performed at 1, 2 and 3 weeks of age. No difference in apoptosis was observed between WT and B6-*Sox3*$^{-/Y}$ mice at any time point examined (not shown), suggesting that the absence of mature germ cells at 2 weeks reflects a defect in differentiation of the spermatogonia.

Sox3 is localized in undifferentiated spermatogonia

To further characterize the role of Sox3 in germ cell function, the protein was localized by immunohistochemistry in postnatal testis. Beginning at P7, intense nuclear Sox3 staining was observed in a subset of prospermatogonia located adjacent to the basal lamina. At this stage of development, the gonocytes exit cell cycle arrest and enter a wave of proliferation. Staining with Gcna1 confirmed that Sox3 expression was confined to a subset of germ cells (Fig. 5, top left, arrows). Sox3 expression decreased in adult animals and was confined to selected spermatogonia distributed sparsely on the periphery of the seminiferous tubule, independent of the epithelial cycle (Fig. 5, top right). Co-expression of Gcna1 with Sox3 was not observed in adult tissue, when Gcna1 becomes restricted to differentiated spermatogonia. However, adult Sox3-positive cells exhibited a flattened surface resting on the basal lamina and a rounded surface in contact with the Sertoli cell, morphological characteristics that are compatible with type A spermatogonia and distinct from Sertoli or peritubular myoid cells (Chiarini-Garcia and Russell, 2001).

Dual staining was also performed using antibodies to Sox3 and the proliferation marker Pcna1 or the meiotic marker γH2aX. At P7, and in adult testis, Sox3 expression is restricted to proliferating spermatogonia (Fig. 5, bottom panels, arrows) and is not expressed in meiotic spermatocytes (not shown). Combined, these results indicate that Sox3 is expressed specifically in type A (A_s, A_{pr} and A_{al}) spermatogonia.

Sox3 acts through neurogenin 3 to promote spermatogenesis

Neurogenin 3 (Ngn3) is a transcription factor of the basic helix–loop–helix (bHLH) family that is expressed in neural and endocrine precursor cells (Gradwohl et al., 2000). Recent experiments demonstrate that Ngn3 is also expressed specifically in A_s, A_{pr} and A_{al} spermatogonia (Yoshida et al., 2004), where it delineates the earliest stages of spermatogenesis. In WT mice from the B6-*Sox3* colony, Ngn3-positive spermatogonia were detectable at 1 week after birth (Fig. 6A). Ngn3 staining decreased over time but was still clearly detectable at 3 weeks after birth. Faint Ngn3 staining was also observed in some spermatocytes.

Double immunofluorescence showed complete overlap between Sox3-positive cells and Ngn3 expression at 1, 2 and 3 weeks after birth (white arrowheads). These data confirm that Sox3 expressing cells are A_s, A_{pr} and A_{al}

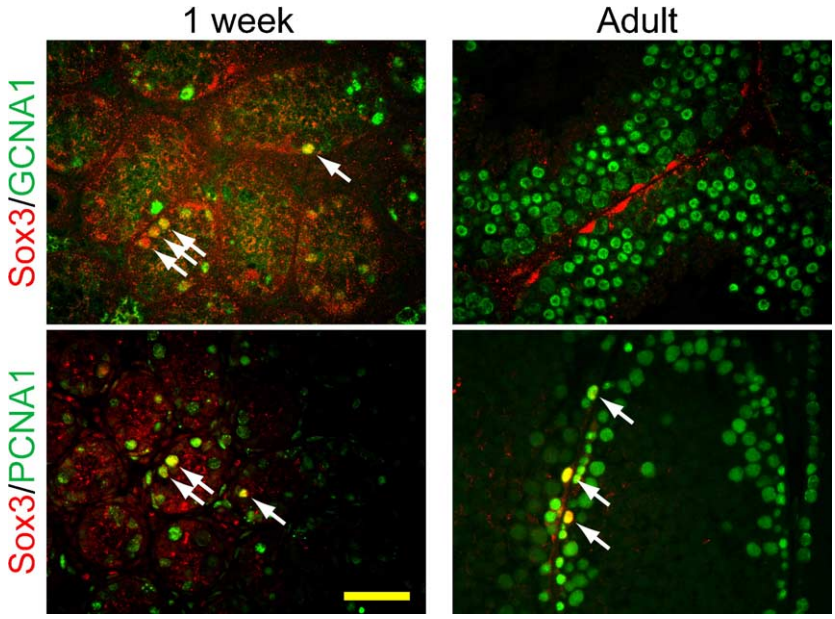

Fig. 5. Expression of Sox3 in undifferentiated spermatogonia. Double immunofluorescence using antibodies to Sox3 (red) and Gcna1 (green, top panel) or Pcna1 (green, bottom panel). Expression of Sox3 was detected in spermatogonia at 1 week of age. Colocalization of Sox3 with Gcna1 was observed at 1 week (yellow), and colocalization of Sox3 with Pcna1 was observed beginning at 1 week and continuing through the adult. Scale bar = 50 μm.

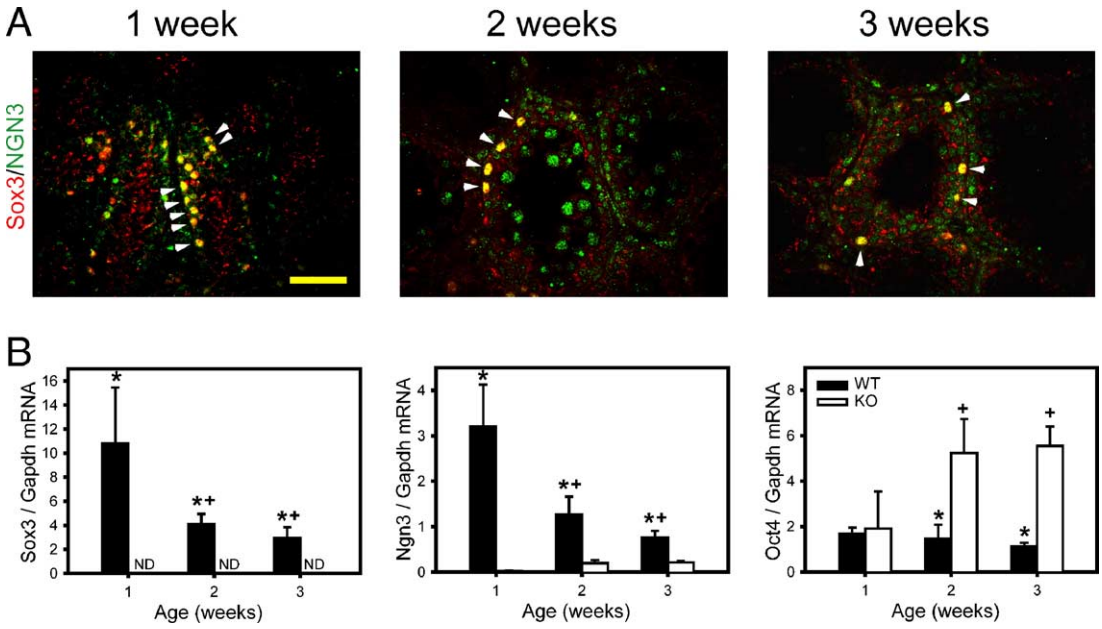

Fig. 6. Sox3 and Ngn3 during postnatal weeks 1, 2 and 3. (A) Double immunofluorescence in WT mice using antibodies to Sox3 and Ngn3. Note co-expression in undifferentiated spermatogonia. Scale bar = 50 μm. (B) Gene expression patterns in WT and B6-*Sox3*$^{-/Y}$ mice. Note the decrease in *Ngn3* and increase in *Oct4* expression in the absence of *Sox3*. *Sox3*, *Ngn3* and *Oct4* mRNAs are calculated as a ratio to Gapdh and presented as arbitrary units. Asterisk (*) indicates $P < 0.05$, WT versus KO ($n = 3$). Plus sign (+) indicates $P < 0.05$ versus 1 week time point of the same genotype. ND, not detected.

spermatogonia. Furthermore, expression of Sox3 and Ngn3 in the same cells suggests a potential functional link between these two transcription factors.

To explore this possibility further, levels of *Sox3* and *Ngn3* mRNAs were measured in WT and B6-*Sox3*$^{-/Y}$ mice. In WT mice, *Sox3* mRNA levels were high at 1 week and gradually declined with the progression of spermatogenesis (Fig. 6B). This pattern was mirrored by *Ngn3* mRNA, consistent with a previous report (Yoshida et al., 2004). Of particular interest, *Ngn3* expression was abolished in the absence of *Sox3*. The absence of Ngn3 expression does not appear to be due to cell loss, as levels of Oct4 mRNA were increased in B6-*Sox3*$^{-/Y}$ mice. Oct4 is a marker of undifferentiated cells, including spermatogonia (Pesce et al., 1998). Supporting this conclusion, we also observed persistence of the germ cell markers c-ret (Supplementary Fig. 1) and Stra8 (Menke et al., 2003; Viglietto et al., 2000; Widenfalk et al., 2000) (data not show). Taken together, these data demonstrate that *Ngn3* expression is dependent on Sox3.

Discussion

In this study, we demonstrate that Sox3 is expressed in type A spermatogonia and that loss of Sox3 is associated with failure of germ cell maturation. Previous studies implicated Sox3 in spermatogenesis, although the mechanism was unclear. Rizzoti et al. (2004) suggested that reduced testis size and impaired spermatogenesis in the absence of Sox3 were due to pituitary dysfunction. However, we find that pituitary gonadotropin levels and testosterone production are similar in WT and *Sox3* null mice, indicating a primary testis defect. In a previous study, we proposed that germ cell loss might be secondary to Sertoli cell dysfunction (Weiss et al., 2003). The current data, however, suggest that the loss of Sox3 affects spermatogenesis more directly by blocking the differentiation of spermatogonia.

The Sox family of proteins plays a critical role in testis development and function. Sry is necessary and sufficient to induce testis development from the undifferentiated gonad (Koopman et al., 1991; Sinclair et al., 1990). Sry is expressed transiently in the progenitor cells that give rise to the Sertoli cells. Soon after its expression, Sox9 is expressed in Sertoli cells. It, too, is sufficient to induce testis development in XX transgenic mice engineered to express Sox9 (Vidal et al., 2001). Although Sox3 shares DNA binding characteristics with Sry and Sox9 (Bergstrom et al., 2000), it is expressed predominantly, if not exclusively, in the germ cells rather than somatic cells of the gonad. While we cannot exclude low level expression in somatic cells, Sertoli function appears preserved in the developing testis of the Sox3 null mouse.

The importance of genetic background in the study of gonadal development is well established (Albrecht et al., 2003). We chose to examine the effect of Sox3 deletion on the C57/BL6 background, as it resulted in a more pronounced and consistent defect in testis function. By comparison to 129 background, adult B6-*Sox3*$^{-/Y}$ had smaller testes and more extensive loss of germ cells, leading to complete infertility. Because of these features, it was

possible to more clearly identify the timing of germ cell loss, which began between P7 and P10, a time when spermatogonial differentiation is initiated.

It has been challenging to establish the cellular location of Sox3 in the testis. The earliest studies of Sox3 expression were performed using RNAse protection (Collignon et al., 1996). These studies documented *Sox3* mRNA in the genital ridge of germ cell deficient mice at E11.5, suggesting that Sox3 is expressed only in somatic cells. Expression levels were extremely low, however, and the mouse model used may retain a small number of germ cells. A later study using in situ hybridization identified *Sox3* expression within the developing testis cords at E16.5 and in the young adult testis (p35). The primary conclusion was that *Sox3* is not expressed in the interstitial Leydig cells. The authors suggested that *Sox3* is expressed in the Sertoli cells, but it should be noted that, while *Sox3* appeared to be restricted to the basal compartment of the seminiferous tubules that houses the Sertoli and undifferentiated germs cells, cells expressing *Sox3* were not identified by dual staining with cell type specific markers (Shen and Ingraham, 2002).

In a previous study, we suggested, based on immunochemistry and cellular morphology, that Sox3 was expressed in Sertoli cells. In a more recent paper, Sox3 immunohistochemistry confirmed the localization of Sox3 to the basal compartment, but the authors concluded based on nucleolar morphology that the cells expressing Sox3 were not somatic cells (Rizzoti et al., 2004) but rather germ cells. To address this issue directly, we co-stained postnatal and adult testis for Sox3 and known markers of somatic and germ cells (Figs. 4 and 5). These data provide strong evidence that Sox3 expression is restricted to type A spermatogonia, which includes the stem cell population (A_s) that supports continuous spermatogenesis in addition to germ cells in the earliest stages of differentiation (A_{pr}, A_{al}). Consistent with these findings, *Sox3* is expressed in a cluster of primordial germ cells in the avian embryo (Uchikawa et al., 1999), suggesting that *Sox3* may be expressed at multiple time points or even throughout germ cell development.

Although *Sox3* mRNA can be detected by RT-PCR in embryonic mouse gonads as early as E11.5 (unpublished data), we have been unable to detect Sox3 protein in these embryos using the same antibody that was successful in postnatal testis. It is possible that embryonic *Sox3* mRNA may not be efficiently translated, or protein levels may be too low to be detected by antibody. Targeted deletion of *Sox3* had no apparent effect on the migration or eventual number of germ cells (unpublished data) nor did we observe any defect in the development of the gonad or its constituent cell populations in the absence of Sox3 (Fig. 2). Thus, a functional role for Sox3 in the embryo remains to be demonstrated.

In the absence of an embryonic defect, we focused on the postnatal period. The most striking result was the extensive failure of germline stem cells to differentiate in the absence of Sox3. Defects were observed as early as P10, and by 14 days after birth, only Sertoli cells and undifferentiated spermatogonia remained in the seminiferous tubules of knockout mice. The timing of this failure is potentially informative, as it coincides with initiation of the first meiosis. Leptotene spermatocytes, which represent cells in the earliest stage of meiosis, are normally observed for the first time between P8 and P10. In *Sox3* knockout animals, spermatocytes at any stage are absent from severely affected tubules. These results suggest that Sox3 may be involved in differentiation of spermatogonia or in the initiation or early progression of meiosis, though additional experiments will be required to test this hypothesis.

In considering mechanisms through which Sox3 might act, it is provocative to consider the role of Sox proteins in neuronal development. Sox genes from the B1 subgroup (*Sox1*, *Sox2* and *Sox3*) are expressed throughout the neural primordium. *Sox1–3* are specifically expressed in neural progenitor cells, as evidenced by co-expression with neurogenin 2 (*Ngn2*), a proneural transcription factor of the bHLH family (Bylund et al., 2003). Group B1 *Sox* genes have been shown to act in a redundant fashion to oppose neurogenesis and maintain neural progenitor cells in an undifferentiated state (Bylund et al., 2003; Graham et al., 2003). However, a different study concluded that *Sox1*, but not *Sox2* or *Sox 3*, instead promote neurogenesis through a mechanism that involves induction of *Ngn1* (Kan et al., 2004).

In light of these experiments, we were intrigued by the recent demonstration that *Ngn3* is specifically expressed in undifferentiated spermatogonia (A_s, A_{pr} and A_{al}) (Yoshida et al., 2004). Spermatogonial stem cells share some functional characteristics with other stem cells (Kubota et al., 2003), and we hypothesized that similar interactions might occur between *Sox3* and *Ngn3* to regulate germ cell differentiation and/or the control of spermatogenesis. Data presented in Fig. 6 provide support for this hypothesis. In addition to colocalization of *Sox3* and *Ngn3*, *Ngn3* expression was highly dependent on the presence of *Sox3*. This observation suggests a functional relationship between these proteins similar to that observed for Sox proteins and neurogenins 1 and 2 in neuronal stem cells.

Nonetheless, the specific role of Ngn3 in spermatogenesis has not been determined. While data in this manuscript and published reports define a temporal window in which Sox3 might act, several mechanisms for Sox3 action remain plausible. For example, Sox3 might act directly, or through Ngn3, to control spermatogenic stem cell fate or the success of spermatogonial differentiation. Loss of Ngn3 expression might also set the stage for later spermatogenic failure. Alternatively, Ngn3 may not be causally involved in the Sox3 phenotype. Additional experiments focusing on this period of development will be required to distinguish among these possibilities.

In addition, we observed upregulation of *Oct4* gene expression in the absence of *Sox3*. *Oct4* is a marker of undifferentiated cells, including spermatogonia. The signifi-

cance of this change in gene expression is somewhat difficult to assess, as the loss of more differentiated germ cells in the Sox3 KO could increase the number of *Oct4*-expressing cells relative to other testis cells, and thereby to the Gapdh control mRNA, in the absence of any true change in *Oct4* expression. Of note, however, the level of *c-Ret* mRNA, which is specific to spermatogonia, is similar between WT and KO animals at 2 weeks and increases in the KO at 3 weeks, a period over which there is significant loss of differentiated germ cells. Thus, it is possible that the apparent increase in *Oct4* expression in the Sox3 KO reflects a transient expansion in the pool of undifferentiated spermatogonia or that Sox3 regulates Oct4 directly in the absence of any change in cell number. *Oct4* may also play an active role in promoting germ cell survival, contributing further to the increase in levels of *Oct4* mRNA (Kehler et al., 2004).

In conclusion, we have localized *Sox3* expression to the undifferentiated spermatogonia and identified a role for *Sox3* in the initiation or progression of spermatogenesis. *Sox3* is one of a small number of factors identified to date that are required at the earliest stages of germ cell maturation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2005.04.013.

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