Published in final edited form as:
Dev Biol. 2008 April 15; 316(2): 359–370.

Sox8 is a critical regulator of adult Sertoli cell function and male fertility

Moira K. O'Bryan[1,2], **Shuji Takada**[3], **Claire L. Kennedy**[1,2], **Greg Scott**[4], **Shun-ichi Harada**[7], **Manas K. Ray**[4], **Qunsheng Dai**[6], **Dagmar Wilhelm**[3], **David M. de Kretser**[1,2], **E. Mitch Eddy**[6], **Peter Koopman**[2,3], and **Yuji Mishina**[4,5]

1*Monash Institute of Medical Research, Monash University, Melbourne, Australia*

2*ARC Centre of Excellence in Biotechnology and Development*

3*Institute for Molecular Bioscience, The University of Queensland, Brisbane, Australia*

4*The Knockout Core, National Institute of Environmental Health Sciences, Research Triangle Park, NC, 27709, USA*

5*Molecular Developmental Biology Section, National Institute of Environmental Health Sciences, Research Triangle Park, NC, 27709, USA*

6*Gamete Biology Section, Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, Research Triangle Park, NC, 27709, USA*

7*Department of Bone Biology and Osteoporosis Research, Merck Research Laboratories, West Point, PA, USA.*

Abstract

Sox8 encodes a high mobility group transcription factor that is widely expressed during development. *Sox8*, *−9* and *−10* form group E of the *Sox* gene family which has been implicated in several human developmental disorders. In contrast to other *SoxE* genes, the role of *Sox8* is unclear and *Sox8* mouse mutants reportedly showed only idiopathic weight loss and reduced bone density. The careful analysis of our *Sox8* null mice, however, revealed a progressive male infertility phenotype. *Sox8* null males only sporadically produced litters of reduced size at young ages. We have shown that SOX8 protein is a product of adult Sertoli cells and its elimination results in an age-dependent deregulation of spermatogenesis, characterized by sloughing of spermatocytes and round spermatids, spermiation failure and a progressive disorganization of the spermatogenic cycle, which resulted in the inappropriate placement and juxtaposition of germ cell types within the epithelium. Those sperm that did enter the epididymides displayed abnormal motility. These data show that SOX8 is a critical regulator of adult Sertoli cell function and is required for both its cytoarchitectural and paracrine interactions with germ cells.

Keywords

Testis; fertility; Sox; sperm; adhesion; spermatogenesis

Address for correspondence regarding the mouse model: Yuji Mishina. Ph: 1 919 541 1095; Fax:, 1 919 541 3800; mishina@niehs.nih.gov. Address for correspondence regarding the manuscript: Moira O'Bryan. Ph: 61 3 9594 7407; Fax:, 61 3 9594 7439; moira.obryan@med.monash.edu.au.

Introduction

SOX proteins, exemplified by the mammalian Y-linked sex determination gene *Sry*, are transcription factors that possess a high-mobility group (HMG) domain with the ability to bind and bend DNA, thus allowing the *trans*-activation of target genes (Giese et al., 1994; Pontiggia et al., 1994). *Sox* genes are predominantly, but not exclusively, expressed during embryogenesis and in humans and mice form a family of 20 members (Schepers et al., 2000). Based on differing levels of structural and organizational similarities, and likely evolutionary relatedness, the *Sox* family has been subdivided into 10 subgroups, A-J (Bowles et al., 2000). The *SoxE* group is composed of *Sox8*, *−9* and *−10*, and has been implicated in a number of human disorders including XY sex reversal, campomelic dysplasia (Foster et al., 1994; Wagner et al., 1994), Waardenberg-Hirschsprung disease type IV (aganglionic megacolon) (Kuhlbrodt et al., 1998; Pingault et al., 1998; Southard-Smith et al., 1998), Yemenite deaf-blind hypopigmentation (Bondurand et al., 1999) and chronic intestinal pseudo-obstruction (Pingault et al., 2000). Consistent with a shared evolutionary origin, *SoxE* genes frequently display overlapping expression patterns and have been shown to compensate for, or co-operate with, each other in models of gene mutation. For example, in mouse models of Waardenberg-Hirschsprung disease caused by haploinsufficiency of *Sox10*, ablation of *Sox8* further inhibits enteric neural crest stem cell migration (Maka et al., 2005), and loss of *Sox8* exacerbates the sex-determination defects seen in heterozygous *Sox9* mutants (Chaboissier et al., 2004)

Initially, based on the specific expression in testis cords at 13.5 days post-coitum (dpc) and the ability to induce *Amh* expression in vitro, *Sox8* was proposed as a regulator of male sex determination, testicular differentiation or germ cell development (Schepers et al., 2003; Schepers et al., 2002). However, genetic ablation of *Sox8* in mice failed to result in an abnormal sexual development phenotype. Closer analysis revealed that $Sox8^{-/-}$ mice showed decreased adiposity (Sock et al., 2001), premature osteoblast differentiation which resulted in poor tarsal development and low bone density (Schmidt et al., 2005; Sock et al., 2001). The lack of defects in sex determination have been attributed to functional compensation by other *SoxE* family members (Chaboissier et al., 2004; Koopman, 2005).

To study further the role(s) of SOX8 in mice, we generated a *Sox8* knockout mouse strain. In the course of establishing a colony of these mice, it became apparent that $Sox8^{-/-}$ males rarely produced litters, while $Sox8^{+/-}$ males and $Sox8^{-/-}$ females appeared reproductively normal. In the present study we demonstrate that *Sox8* is essential for the maintenance of male fertility beyond the first wave of spermatogenesis. We found that the loss of SOX8 resulted in progressive degeneration of the seminiferous epithelium through perturbed physical interactions between Sertoli cells and the developing germ cells.

Materials and Methods

Construction of the Targeting Vector and Generation of Sox8 lacZ Knock-in Mice

To generate *Sox8* lacZ knock-in mice, a 2.6kb of *Sox8* locus including exon 1 and 2 was replaced with the lacZ and the neomycin phosphotransferase gene (neo) cassette. Because of the polyA site in the lacZ cassette exon 3 of the *Sox8* locus is unlikely to be expressed. Exon 3 is the last exon in the *Sox8* locus and encodes 248 amino acids of C-terminus of the protein. In addition, loxP (recognition sequence for Cre recombinase) sites were added surrounding the neo cassette for future removal of the neo cassette. To facilitate the homologous recombination, a 4.0kb upstream region of exon 1 was inserted upstream of the lacZ-polyA-neo cassette as a 5' arm, and a 2.8kb 3' flanking region downstream of exon 2 was inserted downstream of the lacZ-polyA- neo cassette as a 3' arm. The final *Sox8* knock-in construct (Fig. 1A) was confirmed by restriction enzyme mapping and sequencing. Twenty five micrograms of linearized targeting vector was electroporated into 10^7 AB2.2 ES cells (Lexicon Genetics) that

were subsequently cultured in the presence of G418 on mitotically inactivated STO fibroblasts. Six hundred G418-resistant ES clones were initially screened by *Eco*RI or *BamH*I digestion and hybridized with unique 5′ or 3’ probes respectively, external to the region of vector homology (Fig. 1B). Sixteen correctly targeted ES clones were identified. Three of the *Sox8*-targeted ES clones were microinjected into C57BL/6 blastocysts (Taconic), and the resulting chimeric embryos were transferred to the uterine horns of day 2.5 pseudopregnant foster mothers. Chimeras were identified among the resulting progeny by their agouti fur and were subsequently bred with C57BL/6 mates. One of the targeted ES clones (3C4) contributed to the germline of chimeric mice. Tail DNA from the agouti pups that resulted from these matings was analyzed by Southern blot to identify *Sox8* heterozygotes. To exclude the possibility that the observed phenotype was related to the insertion of the neo cassette in the targeting construct, the neo cassette was removed by crossing *Sox8* mice with *Mox2*-Cre transgenic mice (Tallquist and Soriano, 2000). The resulting *Sox8* mutant mice were kept on a mixed 129SvEv (20–30%) and C57BL6/Tac (70–80%) background.

Animals were maintained under standardized conditions of lighting (12L:12D) and nutrition (food and water ad libitum). All studies were performed in accordance with the institutional guidelines covering the humane care and use of animals in research at the National Institute of Environmental Health Sciences.

Genotyping

Genotyping of embryos and pups was determined by PCR analysis of genomic DNA, extracted from tail DNA or ear punch, with primers S8a: 5′-GAGGACAAAGATTGGGTCCTGC-3′ and S8b: 5′-GAAGCGTTCGTCTGCTGCC-3′ to detect the wild-type allele (299bp); and primers S8a: 5′- GAGGACAAAGATTGGGTCCTGC-3′ and S8c: 5′-GATGAAACGCCGAGTTAAACGC-3′ to detect the mutant allele (553bp) (Fig. 1D). For confirmation of the deletion of the neo cassette, PCR analyses for 3’ end of the lacZ-polyA-neo cassette was carried out with primers S8d: 5’-TCACTTCGGTGCTTAGTGCTGG-3’ and S8e: 5’-GGCAATAGCAGGACTGATCTGG-3’ to detect the wild-type allele (309bp); primers S8f: 5’-AGGATTGGGAAGACAATAGCAGG-3’ and S8e: 5’-GGCAATAGCAGGACTGATCTGG-3’ to detect the mutant allele (350bp before, and no amplification after removal, of the neo cassette). Deletion of the neo cassette was further confirmed with primers that amplify the neo sequence, Neo-A: 5’-GGATCGGCCATTGAACAAGATGGATTGCAC-3’ and Neo-T: 5’-CCTGATGCTCTTCGTCATCCTGAT-3’ (496 bp before, and no amplification after, removal of the neo cassette) (Fig. 1E).

In order to assess the veracity of the *Sox8* knockout strategy, testes were removed from 10 week old wild-type, heterozygous and knockout animals. RNA was extracted using standard methods and RT-PCR carried out to assess production of the targeted exons (1 and 2), and the remaining exon (exon 3) separately. In addition, the presence of the LacZ transcript, to indicate the presence of a targeted locus, was assessed and *Hprt* expression was also assessed as a control for mRNA quality. The primers used to assess *Sox8* exon 1–2 expression were: ATCCGTGATGCCGTGTCGC (forward) and TGGGTGGTCTTTCTTGTGCTG (reverse). The primers used to assess *Sox8* exon 3 expression were: ACCAGGCGAATGGAAGC (forward) and TGGTAGAGGCTGGGAGGGTA (reverse). The primers used to assess *LacZ* expression were: GCGTTACCCAACTTAATCG (forward) and TGTGAGCGAGTAACAACC. The primers used to assess *Hprt* expression were: CCTGCTGGATTACATTAAAGCACT (forward) and GTCAAGGGCATATCCAACAACAAA (reverse).

Development and function of male fertility

To examine if the failure of spermatogenesis seen in *Sox8*-null mice was derived from the incomplete differentiation of the testis in the embryo, we analyzed by section *in situ* hybridization the expression of marker genes for Sertoli and Leydig cells using anti-Müllerian hormone (*Amh*) and cytochrome P450 side chain cleavage enzyme 11a1 (*Scc*), respectively. Testes and epididymides from 20-day-old, 1-, 2-, 3-, 5- and 9-month-old males were removed, weighed, immediately immersion fixed in Bouin's fluid and processed into paraffin as described previously (O'Bryan et al., 2000). Five μm sections were stained with periodic acid Schiff (PAS) reagent and analyzed for spermatogenic abnormalities. Because of the increasing disorganization of the seminiferous epithelium with age, particular attention was paid to the extent of acrosome development in spermatids and spermatogonial type. Cauda epididymides were collected from 2- and 5-month-old animals and sperm harvested and analyzed using a computer assisted sperm analyzer as described previously (Slott et al., 1993). Parameters measured included path velocity (VAP), progressive velocity (VSL), track speed (VCL), amplitude of lateral head displacement (ALH), beat cross frequency (BCF) and linearity (LIN). In order to compensate for the large quantitative differences in the number of sperm harvested from *Sox8*$^{+/-}$ and *Sox8*$^{-/-}$ epididymides, percentage changes in motility parameters were compared rather than absolute numbers. Caudal epididymal sperm counts were determined as described by Robb et al (Robb et al., 1978). Tissues for electron microscopy were prepared as outlined previously (Miki et al., 2002).

Serum levels of the key reproductive hormones follicle stimulating hormone (FSH) and inhibin were measured by radioimmunoassay as described previously (Kennedy et al., 2005) (n = 31 for wild type animals, and n = 30 for knockout animals).

Protein expression

Espin, Vinculin and SOX8 within the seminiferous epithelium of wild-type testis (SOX8) and knockout testis (Espin and vinculin) was localized using the Dako EnVision method as recommended by the manufacturer following tissue processing and antigen retrieval, as described previously (O'Bryan et al., 2000). SOX8 immunofluorescence was performed on frozen testis as previously described using Oregon Green-conjugated anti-mouse immunoglobulin G (Young et al., 2006). Espin antiserum was obtained from Transduction Laboratories and used at a concentration of 1.25 μg/ml, Vinculin antiserum was obtained from Sigma Laboratories and used at a dilution of 1 in 1000 and an affinity-purified rabbit SOX8 antiserum raised against the peptide HVEDSDSDAPPSPAGE (amino acids 24–38 of mouse SOX8) was used at a 1 in 100 dilution (for both immunohistochemistry and immunofluorescence). The localization of SOX8 within the adult testis was confirmed by LacZ staining of *Sox8*$^{+/-}$ testes as described previously (Mishina et al., 1991). The specificity of the SOX8 serum was determined by Western immunoblotting of adult testis extract from Sox8$^{+/+}$ and Sox8$^{-/-}$. Consistent with the predicted molecular weight of SOX8, the serum bound exclusively to a band in the Sox8$^{+/+}$ testis extract but not the Sox8$^{-/-}$ extract. The specificity of the band was further assured by the binding of the C-19 SOX8 antiserum (Santa Cruz Biotechnology Inc) to the same band. Apoptotic cells were visualized using the ApoTag Plus Peroxidase *In Situ* Apoptosis Detection Kit (Serologicals Corporation) as recommended by the manufacturer.

In order to define the relative levels of SOX8 within the developing post-natal testis, testes were removed from adult and 6, 14, 22, and 30-day-old mice. Protein was extracted and subjected to Western blotting as described previously (Gibbs et al., 2007) using the C-19 serum at a concentration of 200 ng/ml.

Results

Production of *Sox8* knockout mice

A 2.6kb section of the *Sox8* locus, including exons 1 and 2, was replaced with the lacZ expression cassette and the floxed PGK-neo cassette (Figure 1A). Homologous recombination in ES cells was confirmed by Southern blot analyses using both sides of external probes (Figure 1B). Intercrossing of heterozygous mutant mice produced viable homozygous pups (Figure 1C). The floxed neo cassette was removed by crossing with *Mox2*-Cre mice and removal of the cassette was confirmed by genomic PCR (Figure 1D–E). Although there are no significant difference in expression patterns of lacZ and phenotypes in male fertility, we focused on the mutant mice without the neo cassette for further analyses described below. As indicated in Fig. 2A the targeted disruption of the *Sox8* locus resulted in a complete absence of *Sox8* mRNA production. Neither the targeted exons (exon 1 and 2) nor the residual coding exon (exon 3) were expressed in the knockout animals. Absence of the SOX8 protein was confirmed by western blotting (Figure 2B).

Development and function of male fertility

Consistent with previously published data (Chaboissier et al., 2004; Sock et al., 2001), we observed a normal distribution of phenotypically and genotypically male and female embryos with apparently normal testis development in male offspring (data not shown). Similarly, there were no overt differences in the expression level or distribution of *Amh* and *Scc* mRNA in developing gonads (data not shown).

Despite long periods of pairing between adult $Sox8^{-/-}$ males and wild-type females, the majority of pairings did not result in pregnancy. As this male infertility was an unexpected finding, at this early stage mice were only noted as fertile or infertile and pup numbers per plug were not monitored. From these initial pairings, ~80% of $Sox8^{-/-}$ males were sterile, ~20% produced one or more litters, the majority of which were of reduced size. Following the recognition of a male infertility phenotype 8 $Sox8^{-/-}$ males were paired with wild type females from the age of 4–5 weeks of age ie. before sexual maturity. The rationale for doing this was that based on our histological assessment we reasoned that spermtogenesis was more ‘normal’ during the first few waves of the spermatogenic cycle than in later cycles. Thus we suspected fertility would also be higher. Of the 8 young $Sox8^{-/-}$ males tested 3 were sterile and 5 ultimately produced pups. Two produced one litter only, 1 produced 2 litters, and 2 produced 4 litters. With the exception of one litter of 11 pups, all other litters were of reduced size (<7, compared to an average of 10–11 pups per litter in wild-type and heterozygous males) and litter sizes all decreased with increasing age of the father. No fertile $Sox8^{-/-}$ males were observed beyond 150 days of age. These data show that SOX8 is required for fertility in most male mice, but in a percentage of cases (~20%) young animals can sire pups suggesting that at least some sperm resulting from the first few waves of spermatogenesis can be fertile. Female $Sox8^{-/-}$ mice were fertile at all ages examined (data not shown). FSH and inhibin levels, as determined by radioimmunoassay, did not vary between $Sox8^{-/-}$ and *Sox8* control mice (FSH- 4.463±4.715 (null, n = 30), 4.59±1.927 (control, n = 31); inhibin- 8.459±0.3898 (null, n = 30), 8.337±2.886 (control, n = 31)). Seminal vesicle weights were visually assessed as a biomarker of circulating testosterone levels. There were no overt changes in morphometry. A lack of a role for testosterone in this phenotype is consistent with previous studies using testosterone suppression (Beardsley and O'Donnell, 2003).

Analysis of the testis and body weights from $Sox8^{-/-}$ and $Sox8^{+/-}$ mice revealed an age-dependent decrease in absolute testis weights, and relative to body weights, in the homozygous mutant mice (Figure 3). Similar to the Sock et al *Sox8* knockout model there was a slight but significant decrease in body weight in knockout versus control animals at two months of age

(p=0.0392) (Figure 3). Although the difference between controls and mutants was not statistically significant, *Sox8*$^{-/-}$ males showed decreased body weight at older ages (Figure 3). Testis weights were normal in 20- and 35-day-old animals, but by 2 months of age, the testes of *Sox8*$^{-/-}$ mice were significantly smaller than those of control animals (82% of heterozygous mouse testis weights).

Testis weights of wild-type and heterozygous males were not significantly different during the time frame of this study (data not shown). By 5 months of age, *Sox8*$^{-/-}$ testes were 59% the weight of control animals, and by 9 months, *Sox8*$^{-/-}$ testes were 46% those of control animals. An examination of the seminiferous epithelium revealed several histological abnormalities, the incidence of which also increased with age. Specifically these were: increased epithelial vacuolation, strongly suggestive of recent germ cell loss (Figures 4C and 4D); a decreased number of elongating/elongated spermatids (Figures 4C and 4D); disorientation and inappropriate placement of germ cells within the epithelium; and increased elongated spermatid retention (spermiation failure) (Figure 4A versus 4B–D). Spermiation failure was particularly obvious in stage IX tubules and was evident in 1-month-old mice (data not shown), but became progressively more abundant in 2- and 5-month-old *Sox8*$^{-/-}$ animals (Figures 4B and 4C). Although the total number of elongated spermatids was significantly reduced by 9 months of age, virtually all seminiferous tubules from *Sox8*$^{-/-}$ mice contained elongated spermatids (Figure 4D), whereas in wild-type mice elongated spermatids were only visible in stage I–VIII tubules. In contrast to *Sox8*$^{-/-}$ animals, the seminiferous epithelium from 9-month-old wild-type and *Sox8*$^{+/-}$ animals were normal and spermiation failure was rarely observed (Figure 4A).

Sox8$^{-/-}$ mice had a generalized defect in germ cell placement within the seminiferous epithelium. Similarly, *Sox8*$^{-/-}$ animals showed a progressive dysregulation of the seminiferous epithelial cycle. Disorganization was evident at 2 months of age and extreme by 9 months of age (Figures 4B–D). In contrast, 9-month-old wild-type and *Sox8*$^{+/-}$ animals displayed only the classic germ cell associations defined by Russell et al (Russell et al., 1990)(data not shown and Figure 4A). Disorganization was most clearly evident as abnormal spermatid orientation and placement. For example, within *Sox8*$^{+/-}$ stage IX tubules, spermatids (step 9) which had a relatively well-developed acrosome were all orientated with the acrosome pointing towards the basement membrane (Figure 4A, inset). In contrast, stage IX spermatids from *Sox8*$^{-/-}$ testes, which had an equally well-developed acrosome, were orientated randomly within the epithelium (Figure 4B, inset). It was also common to find germ cells at an inappropriate depth within the epithelium or in close juxtaposition to inappropriate cell types. For example, Figure 4C (inset) shows the presence of round spermatids (~step 8) close to the basement membrane within a largely stage X tubule. Stage X tubules do not normally contain round spermatids, nor are they ever positioned close to the basement membrane. Similarly, Figure 4D shows the presence of diplotene spermatocytes and round spermatids within the same tubule cross-section. Such a juxtaposition never occurs in normal mouse spermatogenesis (Russell et al., 1990).

The disruption of the normal cell associations of the seminiferous cycle was confirmed at an ultrastructural level where germ cells from clearly different stages of development in *Sox8*$^{+/-}$ mice were seen within the same tubules of *Sox8*$^{-/-}$ mice. For example, Figure 5A and 5C show round and elongating spermatids in the same tubules. Similarly, germ cells were frequently seen at an inappropriate depth within the epithelium (Figure 5C). Sertoli cell nuclear profiles appeared normal (Figure 5A and 5B), however, the Sertoli cell cytoplasm, particularly in older animals, was vacuolated and had a generalized lacy or swirled appearance (Figure 5A). Of those sperm that were produced, the tail profiles including the axoneme appeared normal (Figure 5D and data not shown).

In order to determine the process by which germ cells were being lost, wild-type, *Sox8*$^{+/-}$ and *Sox8*$^{-/-}$ testes were stained for DNA fragmentation using the TUNEL method. 2 month old *Sox8*$^{-/-}$ testes did not display an obvious increase in apoptotic cells compared to wild-type testes (Figure 6H and 6G), however, there was a suggestion of increased apoptosis in 9-month-old animals (visible also in periodic acid Schiff (PAS) stained sections, Figure 4D). An examination of the epididymides of *Sox8* homozygous mutant animals, however, showed a marked increase in the number of round germ cells within the lumen compared to control animals (Figure 4E versus 4F). Nuclear profiles suggested that many of these cells were spermatocytes and round spermatids. Similarly, epididymidal sections revealed a dramatic decrease in the number of sperm within the lumen (Figure 4E versus 4F). Sloughed round spermatids and spermatocytes were visible within the epididymidal lumen by 1 month of age and maintained until >9 months of age. These data, and declining testis weights, strongly suggest that ablation of *Sox8* resulted in an age-dependent, progressive deregulation of the cycle of the seminiferous epithelium and germ cell development and the loss of germ cell types predominantly by sloughing. Of those cells that reached the stage of germ cell elongation, many (the majority in older animals) failed to spermiate, thus reducing the epididymal sperm count even further.

Protein expression within the adult mouse testis

The localization of SOX8 was examined in adult wild-type mice. SOX8 was expressed exclusively within Sertoli cells as determined by immunohistochemistry and immunofluorescence on wild-type mouse testes using a SOX8-specific antibody (Figures 6A–C) and LacZ staining of *Sox8*$^{+/-}$ testes (figure 6D). The location of SOX8 protein within Sertoli cells varied however, with the stage of the spermatogenic cycle. Specifically, within stage I-IX tubules SOX8 protein displayed a nuclear and cytoplasmic localization (Figures 6A and 6B, black arrows; 6C open arrows), whereas in stage X-XII tubules SOX8 protein was seen only in the Sertoli cell cytoplasm (Figure 6A, blue arrows showing negative nuclei). The nuclear localization of SOX8 was more clearly visualized by immunofluorescence.

Consistent with production by Sertoli cells, SOX8 protein levels as determined by Western blotting was present in the testes of all ages examined (6 days post-natal through to adult), but with relatively higher levels at younger ages (Figure 7). Expression by Sertoli cells is further strengthened in publicly available microarray analyses of post-natal development eg. GDS607 and GDS410 (available through the GEOprofiles interface), which show highest levels of expression in the early post-natal testis and decreasing levels out to adult hood. This is consistent with the relative dilution of a Sertoli cell product during the establishment of spermatogenesis and increasing germ cell complement.

Ectoplasmic specializations in *Sox8*$^{+/-}$ and *Sox8*$^{-/-}$ testes

The sloughing of spermatocytes and round spermatids and a failure of elongated spermatid spermiation were both suggestive of defective Sertoli-germ cell adhesion. In order to investigate this possibility, testis sections were initially stained for the ectoplasmic specialization marker Espin (an actin bundling protein) and Vinculin (a cytoskeletal protein involved in binding actin to the membrane). Espin and Vinculin immunohistochemistry was used as a marker of both the basal (blood-testis barrier) and apical ectoplasmic specializations (Lee and Cheng, 2004; Pfeiffer and Vogl, 1991).The basal and apical ectoplasmic specializations are junction complexes formed between Sertoli cells at the site of the blood-testis barrier and between Sertoli and elongating/elongated spermatids to prevent premature release respectively. Close to the time of sperm release, at stage VII, the apical ectoplasmic specializations are usually dissolved and elongated spermatids remain attached to Sertoli cells via the tubulobulbar complexes. Disengagement of the Sertoli cells and spermiation occurs at stage VIII (for review see (Lee and Cheng, 2004; Russell, 1993). Consistent with previously

published data (Beardsley and O'Donnell, 2003), Espin staining was observed as a line approximately one cell thickness in from the basement membrane in the majority of tubules, marking the blood-testis barrier in *Sox8*$^{+/-}$ and *Sox8*$^{-/-}$ testes (Figure 6E and 6F, open arrows). Staining was also seen surrounding the apical aspect of elongating/elongated spermatids up to and including step 15 marking the point of adhesion between the Sertoli cell and terminally differentiating germ cell (Figure 6E and 6F, white arrows). Both types of staining were observed in *Sox8*$^{-/-}$ animals, however, a dramatic increase in staining was observed in stage IX tubules, in particular, compared to *Sox8*$^{+/-}$ sections (Figure 6F compared to 6E). The majority of this increase was associated with elongated spermatids which had failed to spermiate (Figure 6F, black arrows). Abnormally stained elongated spermatids were in the process of being drawn down to the apical aspect of Sertoli cell nuclei prior to endocytosis (Figure 6F). These data strongly indicate that a failure of apical ectoplasmic specialization resolution in the later stages of elongated spermatid maturation was the reason for the spermiation failure. There was no overt difference in Espin staining of the blood-testis barrier between *Sox8*$^{-/-}$ and control testes. Similar data was obtained for Vinculin staining (Supplementary Figure 1), further strengthening this conclusion.

The frequent failure of ectoplasmic specialization resolution surrounding terminal step elongated spermatids was subsequently confirmed at an electron microscopic level (Figure 5D) where actin bundles which are the defining feature of the apical ectoplasmic specialization can be clearly seen in retained spermatids (Figure 5E). In contrast, and consistent with the immunohistochemical data, Sertoli-Sertoli cell junctions making up the blood-testis barrier appeared normal at an ultrastructural level (Figure 5A and 5B, open arrows).

Sperm motility parameters

Not surprisingly given the testicular histology, caudal epididymal sperm counts were reduced in knockout animals and worsened with age (results not shown). Even in the most severely affected mice, however, some sperm could be found in the epididymal lumen, leaving open the possibility of achieving fertilization. In order to determine their functional attributes, sperm were harvested from the caudal epididymides of 2- and 5-month-old males. In order to avoid bias as a consequence of the age-related decrease in knockout mouse sperm numbers, the proportion of sperm that were motile were compared rather than absolute number of sperm. *Sox8*$^{-/-}$ males displayed an age-dependent decrease in both the percentage of total motile sperm and the percentage of progressively motile sperm (Figure 8A). By 5 months of age, virtually no sperm displayed progressive motility. Interestingly, of those sperm that were moving, the beat frequency, or the number of times a sperm head crossed an imaginary straight line drawn along the sperm path, was not significantly different between *Sox8*$^{+/-}$ and *Sox8*$^{-/-}$ animals (Figure 8B). Collectively, these data suggest that sperm produced by *Sox8*$^{-/-}$ mice showed an age-dependent decrease in propulsive power.

Discussion

Our present analyses demonstrate that SOX8 is a product of Sertoli cells, and while not critically required for testis specification and development, it is critical for the maintenance of adult male fertility. During development and the first wave of spermatogenesis, *Sox8*$^{-/-}$ mice established histologically normal spermatogenesis and produced motile sperm. In young mice, sperm were capable of producing pups (albeit at reduced litter sizes); however, by 5 months of age, *Sox8*$^{-/-}$ mice were sterile, testis weights were significantly reduced by 2-months-of-age as a result of the sloughing of round germ cells, and sperm counts were further reduced as the result of spermiation failure. Of those sperm that did reach the epididymides, many were immotile or incapable of progressive motility. By 5 months of age, virtually no progressively motile (and therefore fertile) sperm were produced and by 9 months there was an extreme

deregulation of the cycle of the seminiferous epithelium. Collectively, these data infer that SOX8-induced trans-activation is a regulator of both Sertoli cell dynamics and their physical interactions with germ cells.

Following the establishment of full spermatogenesis at post-natal day 35 in the mouse (Kramer and Erickson, 1981), each Sertoli cell concurrently supports 4 or 5 different types of germ cell within its depth. In wild-type testes this is visualized as one of 12 different combinations of cells and is referred to as 'the stage' of spermatogenesis (Russell et al., 1990). Germ cells, and in particular elongated spermataids, are dependent on the Sertoli cells for the mechanical force to move them within the depth of the seminiferous epithelium. Even more impressive is the necessity for different mechanical and paracrine interactions with each germ cell within the depth of the Sertoli cell. Each Sertoli cell is capable of forming 4 or 5 different and ever-changing microenvironments simultaneously around germ cells. *Sox8*$^{-/-}$ mice showed an age-dependent loss of this ability, which by 2 months resulted in signs of spermiation failure and inappropriate germ cell placement, by 5 months of age resulted in sterility and by 9 months of age, a complete loss of the cycle of the seminiferous epithelium.

Deletion of *Sox8* resulted in an age-dependent loss of some, but not all adult Sertoli cell functions. *Sox8*$^{-/-}$ mice were capable of producing both the blood-testis barrier and the apical ectoplasmic specializations holding elongating and elongated spermatids in place. The latter junctions are critically required for the germ cell migration that take place between step 13 and 15 where elongated spermatids are normally drawn towards the apical aspect of the Sertoli cell nucleus, then between steps 15 and 16 where they are pushed towards the tubule lumen prior to release by spermiation. The disengagement of structurally mature sperm from the Sertoli cells occurs in two distinct phases. In the hours preceding spermiation, the apical ectoplasmic specializations (containing Espin and Vinculin) are removed, but germ cells remain tethered to Sertoli cells by the tubulobulbar complexes and junction complexes containing .β1-integrin and β-catenin, but not Espin or Vinculin (reviewed in (Lee and Cheng, 2004). Sperm are physically released following the subsequent removal of the integrin and catenin containing junctional complexes and the disengagement of the tubulobulbar complexes (Beardsley and O'Donnell, 2003). *Sox8*$^{-/-}$ animals displayed an age-dependent, progressive failure of apical ectoplasmic specialization dissolution (ie. the next to final step of sperm release), and as such, sperm where not released into the tubule lumen. Abnormally retained sperm in the process of being phagocytosed by Sertoli cells were increasingly seen in the seminiferous tubule epithelium at all stages of spermatogenesis. In contrast, there was no evidence of abnormal blood-testis barrier formation in *Sox8*$^{-/-}$ animals and germ cells were crossing this barrier indicating appropriate regulation, indicating that Espin expression was not globally perturbed.

The failure of ectoplasmic specialization resolution has not previously been reported and is distinctly different and earlier than the spermiation failure seen following testosterone and FSH suppression. Spermiation failure following hormone suppression is mediated through a failure of tubulobulbar retraction and Sertoli cell disengagement following normal ectoplasmic specialization resorption (Beardsley and O'Donnell, 2003). These data indicate that SOX8 may be a key regulator of the early events of spermiation. Interestingly, abnormal Sertoli-germ cell structures are a common feature in human infertility (Cameron and Griffin, 1998).

Similarly, the attachment of Sertoli cells to spermatocytes and round spermatids in *Sox8*$^{-/-}$ animals was compromised (weakened) and vast numbers of cells were inappropriately sloughed and could be seen within the epididymal lumen. Unlike elongating/elongated spermatids, these cells are usually held in place by a combination of desmosome-like junctions and gap junctions (Lee and Cheng, 2004). These junctions do not contain Espin. The data presented herein strongly suggests that SOX8 *trans*-activation in Sertoli cells has a significant influence on the regulation of Sertoli-germ cell junctions.

In addition to the inferred role in regulating adhesion between Sertoli and germ cells, the data presented suggests that SOX8-regulated genes are important in the paracrine interaction between these two cell types. This is evidenced by the observation that even in those sperm that were produced and spermiated correctly, there was an age-dependent decline in the ability for progressive motility. The possibility remains, however, that SOX8 has a role in sperm maturation via the epididymal lumen.

Another *Sox8* knockout model was reported as having normal male fertility (Sock et al., 2001). Several explanations exist to explain this apparent difference between the two models. The first is that male fertility in the previous models may have been only tested in young animals where some residual fertility remained. The second possibility is that differences in diet underlie the differential effect on Sertoli cell function. The third is that mice were of a different genetic background ie. pure 129Sv (Sock et al., 2001) versus mixed 129SvEv and C57Bl6/Tac herein. The forth possibility is that the difference of the structure of targeted locus. Exon 1, exon 2 and 5' half of exon 3 of *Sox8* was replaced by a lacZ expression cassette with a Neo cassette in the Sock et al model (Sock et al., 2001), whereas exon 3 remained intact in our case. As outlined previously, the chance of the C-terminal portion of SOX8 being produced in our model was minimal as a consequence of the position of the polyA site of the lacZ cassette and a complete absence of exon 3 transcription. Of these possible explanations, we believe differences in background stain is the most likely.

Perhaps one of the biggest questions raised by this study is why the phenotype is age-dependent. An age-dependent fertility phenotype has been reported for several genes and there is good evidence to suggest that the first wave of spermatogenesis is regulated in a different manner to subsequent waves (Falender et al., 2005; Print et al., 1998). As such, the possibility exists that SOX8 truly does become more important for normal function in the older Sertoli cell. Spermatogenesis is an ongoing developmental process and unlike many of the developmental processes in which *Sox8* has been examined (Chaboissier et al., 2004; Hong and Saint-Jeannet, 2005; Schepers et al., 2003), its absence cannot be completely compensated for by *Sox9*. *Sox9* has previously been reported in post-natal Sertoli cells (Foster et al., 1994). The results presented herein raise the possibility of subtle differences in the role of these 2 SoxE genes in spermatogenesis (Kennedy et al., 2007).

The data presented herein clearly demonstrate that SOX8 is a transcription factor with a role in the maintenance of male fertility. SOX8-initiated transcription in Sertoli cells results in the production of a set of molecules which are essential for the maintenance of spermatogenesis and normal sperm function. This model raises the hypothesis that SOX8 is a regulator of Sertoli-germ cell adhesion, and in the setting of the cycle of the seminiferous epithelium (Kennedy et al., 2007). It will be of interest to identify molecular targets of SOX8 and to determine if *SOX8* mutations are involved in hypospermatogenesis phenotypes frequently seen in men.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Toni Ward for assistance with the mouse activity; Ijeoma Nwosu, Leigh E. Davis, Gabrielle Douglas and Jo Merriner for their excellent technical assistance; Tetsuo Kunieda and Peter Stanton and Liza O'Donnell for helpful discussion.

This research was supported by the Intramural Research Program of the NIH, National Institute of Environmental Health Sciences (ES071003-10) the National Health and Medical Research (NHMRC) and the Australian Research Council.

The authors would like to dedicate this paper to Dr. Shun-ichi Harada who loved people and science, and made significant contribution of the field of bone and developmental biology. This work was partially funded by the National Institutes of Health, The Australian Research Council and the Australian National Health and Medical Research Council.

References

Beardsley A, O'Donnell L. Characterization of normal spermiation and spermiation failure induced by hormone suppression in adult rats. Biol. Reprod 2003;68:1299–1307. [PubMed: 12606480]

Bondurand N, et al. A molecular analysis of the yemenite deaf-blind hypopigmentation syndrome: SOX10 dysfunction causes different neurocristopathies. Hum. Mol. Genet 1999;8:1785–1789. [PubMed: 10441344]

Bowles J, et al. Phylogeny of the SOX family of developmental transcription factors based on sequence and structural indicators. Dev. Biol 2000;227:239–255. [PubMed: 11071752]

Cameron, D.; Griffin, F. Ultrastructure of Sertoli-germ cell interactions in the normal and pathologic testis. In: M-GF, R., editor. Male Reproduction: A multidisciplinary overview. Spain: Churchill; 1998. p. 229-242.

Chaboissier MC, et al. Functional analysis of Sox8 and Sox9 during sex determination in the mouse. Development 2004;131:1891–1901. [PubMed: 15056615]

Falender AE, et al. Maintenance of spermatogenesis requires TAF4b, a gonad-specific subunit of TFIID. Genes Dev 2005;19:794–803. [PubMed: 15774719]

Foster JW, et al. Campomelic dysplasia and autosomal sex reversal caused by mutations in an SRY-related gene. Nature 1994;372:525–530. [PubMed: 7990924]

Gibbs GM, et al. Cysteine-rich secretory protein 2 binds to mitogen-activated protein kinase kinase kinase 11 in mouse sperm. Biol Reprod 2007;77:108–114. [PubMed: 17377140]

Giese K, et al. Distinct DNA-binding properties of the high mobility group domain of murine and human SRY sex-determining factors. Proc. Natl. Acad. Sci. U.S.A 1994;91:3368–3372. [PubMed: 8159753]

Hong CS, Saint-Jeannet JP. Sox proteins and neural crest development. Semin Cell Dev Biol 2005;16:694–703. [PubMed: 16039883]

Kennedy CL, et al. Sox8 and Sertoli Cell function. Ann N Y Acad Sci. 2007online publication

Kennedy CL, et al. A repository of ENU mutant mouse lines and their potential for male fertility research. Mol Hum Reprod 2005;11:871–880. [PubMed: 16421219]

Koopman P. Sex determination: a tale of two Sox genes. Trends Genet 2005;21:367–370. [PubMed: 15949865]

Kramer JM, Erickson RP. Developmental program of PGK-1 and PGK-2 isozymes in spermatogenic cells of the mouse: specific activities and rates of synthesis. Dev. Biol 1981;87:37–45. [PubMed: 7286419]

Kuhlbrodt K, et al. Functional analysis of Sox10 mutations found in human Waardenburg-Hirschsprung patients. J. Biol. Chem 1998;273:23033–23038. [PubMed: 9722528]

Lee NP, Cheng CY. Ectoplasmic specialization, a testis-specific cell-cell actin-based adherens junction type: is this a potential target for male contraceptive development? Hum. Reprod. Update 2004;10:349–369. [PubMed: 15192055]

Maka M, et al. Identification of Sox8 as a modifier gene in a mouse model of Hirschsprung disease reveals underlying molecular defect. Dev. Biol 2005;277:155–169. [PubMed: 15572147]

Miki K, et al. Targeted disruption of the Akap4 gene causes defects in sperm flagellum and motility. Dev. Biol 2002;248:331–342. [PubMed: 12167408]

Mishina Y, et al. Thymidylate stress induces homologous recombination activity in mammalian cells. Mutat. Res 1991;246:215–220. [PubMed: 1824719]

O'Bryan MK, et al. Bacterial lipopolysaccharide-induced inflammation compromises testicular function at multiple levels in vivo. Endocrinology 2000;141:238–246. [PubMed: 10614644]

Pfeiffer DC, Vogl AW. Evidence that vinculin is co-distributed with actin bundles in ectoplasmic ("junctional") specializations of mammalian Sertoli cells. Anat Rec 1991;231:89–100. [PubMed: 1750714]

Pingault V, et al. SOX10 mutations in patients with Waardenburg-Hirschsprung disease. Nat. Genet 1998;18:171–173. [PubMed: 9462749]

Pingault V, et al. Peripheral neuropathy with hypomyelination, chronic intestinal pseudo-obstruction and deafness: a developmental "neural crest syndrome" related to a SOX10 mutation. Ann. Neurol 2000;48:671–676. [PubMed: 11026454]

Pontiggia A, et al. Sex-reversing mutations affect the architecture of SRY-DNA complexes. Embo J 1994;13:6115–6124. [PubMed: 7813448]

Print CG, et al. Apoptosis regulator bcl-w is essential for spermatogenesis but appears otherwise redundant. Proc. Natl. Acad. Sci. U.S.A 1998;95:12424–12431. [PubMed: 9770502]

Robb GW, et al. Daily sperm production and epididymal sperm reserves of pubertal and adult rats. J Reprod Fertil 1978;54:103–107. [PubMed: 712697]

Russell, L.; Griswold, M. Role in spermiation. In: Russell LD, GM., editor. The Sertoli Cell. Clearwater, FL: Cache river press; 1993. p. 269-302.

Russell, LB., et al. Histological and Histopathological Evaluation of the testis. New York: Cache River Press; 1990.

Schepers G, et al. SOX8 is expressed during testis differentiation in mice and synergizes with SF1 to activate the Amh promoter in vitro. J. Biol. Chem 2003;278:28101–28108. [PubMed: 12732652]

Schepers GE, et al. Cloning and characterisation of the Sry-related transcription factor gene Sox8. Nucleic Acids Res 2000;28:1473–1480. [PubMed: 10684944]

Schepers GE, et al. Twenty pairs of sox: extent, homology, and nomenclature of the mouse and human sox transcription factor gene families. Dev. Cell 2002;3:167–170. [PubMed: 12194848]

Schmidt K, et al. The high mobility group transcription factor Sox8 is a negative regulator of osteoblast differentiation. J. Cell Biol 2005;168:899–910. [PubMed: 15753123]

Slott VL, et al. Optimization of the Hamilton-Thorn computerized sperm motility analysis system for use with rat spermatozoa in toxicological studies. Fundam Appl Toxicol 1993;21:298–307. [PubMed: 8258383]

Sock E, et al. Idiopathic weight reduction in mice deficient in the high-mobility-group transcription factor Sox8. Mol. Cell Biol 2001;21:6951–6959. [PubMed: 11564878]

Southard-Smith EM, et al. Sox10 mutation disrupts neural crest development in Dom Hirschsprung mouse model. Nat. Genet 1998;18:60–64. [PubMed: 9425902]

Tallquist MD, Soriano P. Epiblast-restricted Cre expression in MORE mice: a tool to distinguish embryonic vs. extra-embryonic gene function. Genesis 2000;26:113–115. [PubMed: 10686601]

Wagner T, et al. Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the SRY-related gene SOX9. Cell 1994;79:1111–1120. [PubMed: 8001137]

Young N, et al. Effect of disrupted SOX18 transcription factor function on tumor growth,vascularization, and endothelial development. J Natl Cancer Inst 2006;98:1060–1067. [PubMed: 16882943]

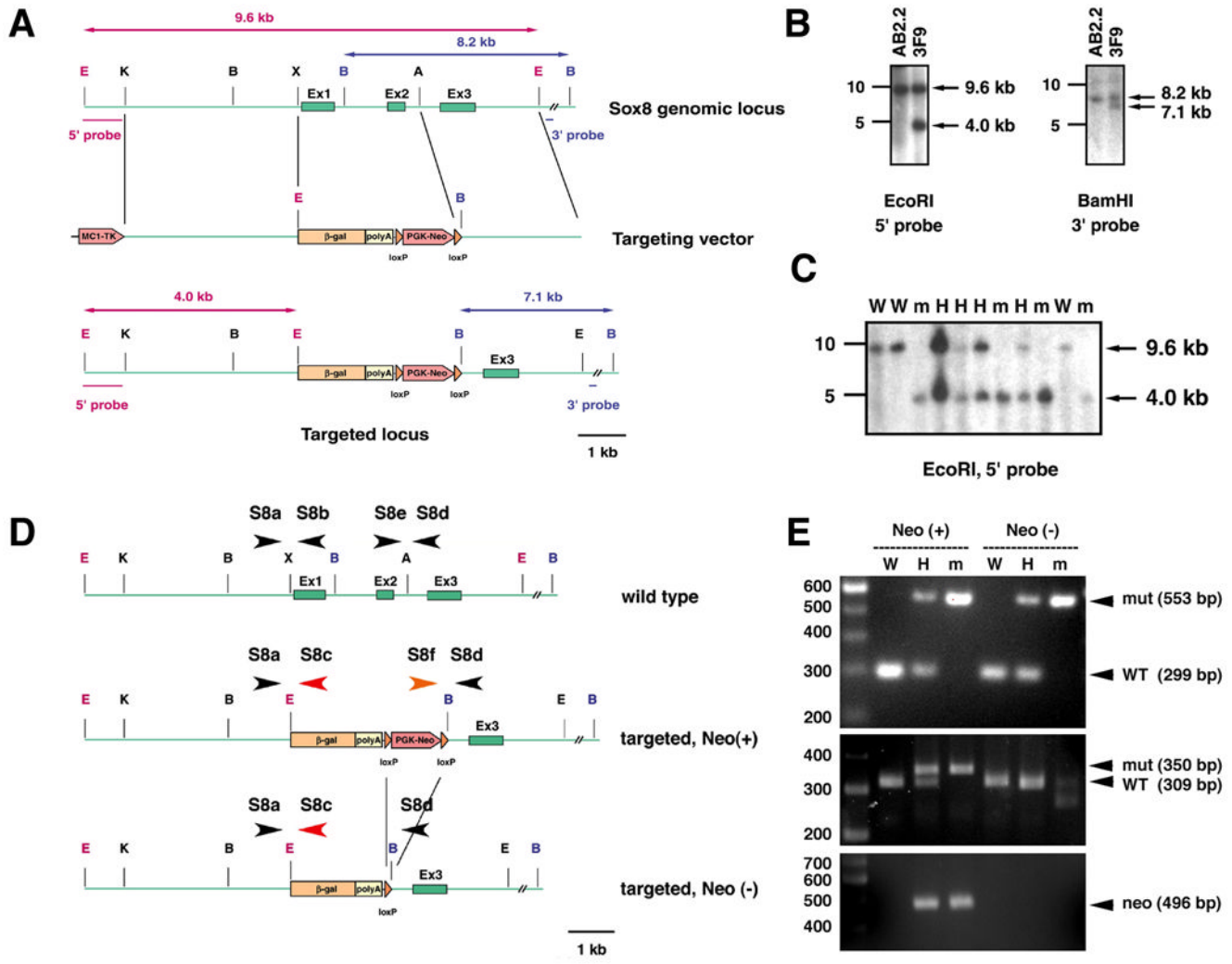

Figure 1.

Generation of lacZ knock in mouse for Sox8. A. Targeting construct. An XbaI-AccI 2.6kb fragment containing exons 1 and 2 was replaced by a lacZ expression cassette followed by a floxed Pgk-neo cassette. Positions of 5' and 3' external probes for Southern analyses and expected band sizes are shown. A, *Acc*I; B, *Bam*HI; RI, *Eco*RI, K, *Kpn*I; X, *Xba*I. B. Confirmation of targeting event in ES cells. Genomic DNA from wild-type ES cells (AS2.2) and correctly targeted cells (clone 3F9) were digested with an enzyme and probed as indicated. C. Interbreeding of heterozygous Sox8 mutant mice generated homozygous mutant mice. All pups from one litter were genotyped at the stage of weaning. D. Schematic representation of structure of each allele and positions of genotyping primers. Primers S8a, S8b, and S8c were used to differentiate the lacZ knock-in allele from WT, whereas primers S8d, S8e and S8f were used to differentiate the lacZ knock-in allele from the lacZ knock-in allele without the neo cassette. E. Examples of genotyping results. Combination of S8a, S8b and S8c differentiated all three genotype regardless the presence of the neo cassette (top). Combination of S8d, S8e, and S8f differentiated the lacZ knock-in allele with the neo cassette from the lacZ knock-in allele without the cassette (middle). Removal of the neo cassette was confirmed with primers that amplified inside of the cassette (bottom). W, wild type; H, heterozygous mutant; m, homozygous mutant. (Sox8 wildtype) $Sox8^{-/-}$ (knockout).

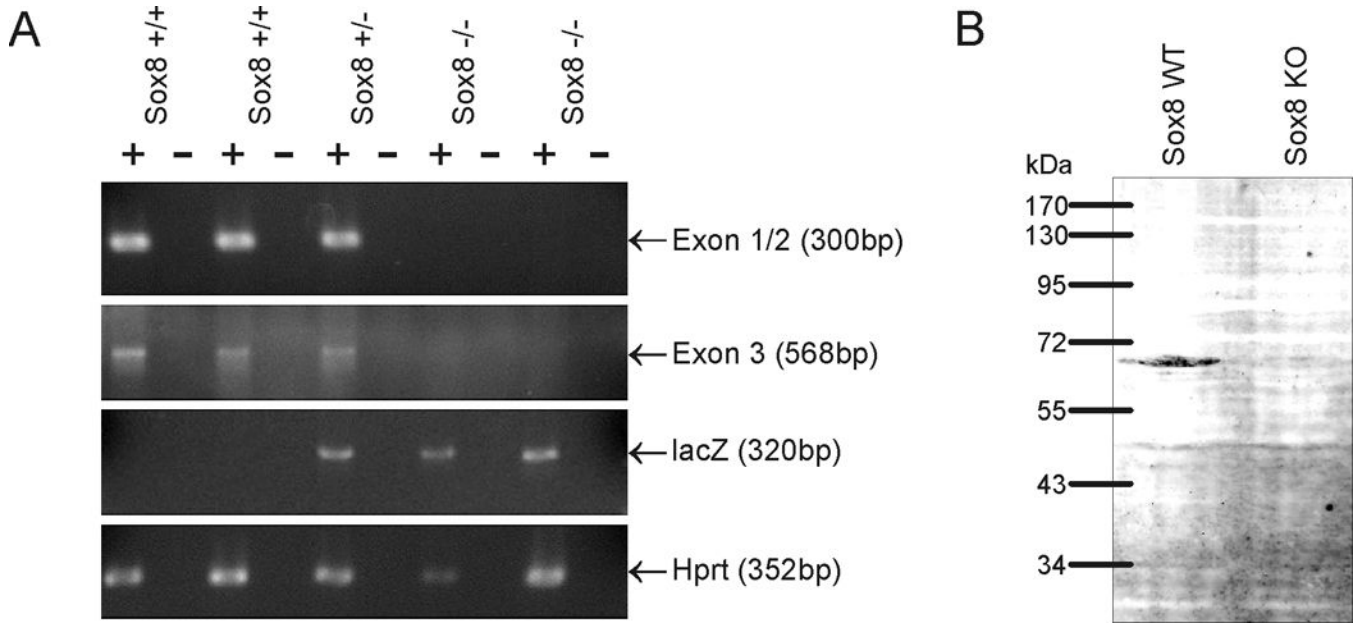

Figure 2.

A. An assessment of Sox8 mRNA production in wild-type (WT), heterozygous (Het) and knockout (mut) mice. Testicular mRNA was extracted from 10 week old animals and analyzed by RT-PCR to assay for cDNA derived from exon 1–2 (targeted exons), cDNA derived from exon 3 which remained *in situ* and LacZ expression (which was targeted into the *Sox8* locus). mRNA quality was assessed using Hprt expression. Product size is indicated in the brackets and RT (+) and no RT (−) is marked. B. The specificity of the anti-SOX8 serum as determined by Western blotting of adult testis extract from $Sox8^{+/+}$ and $Sox8^{-/-}$ animals, showing SOX8 expression in the $Sox8^{+/+}$ but not $Sox8^{-/-}$ animals.

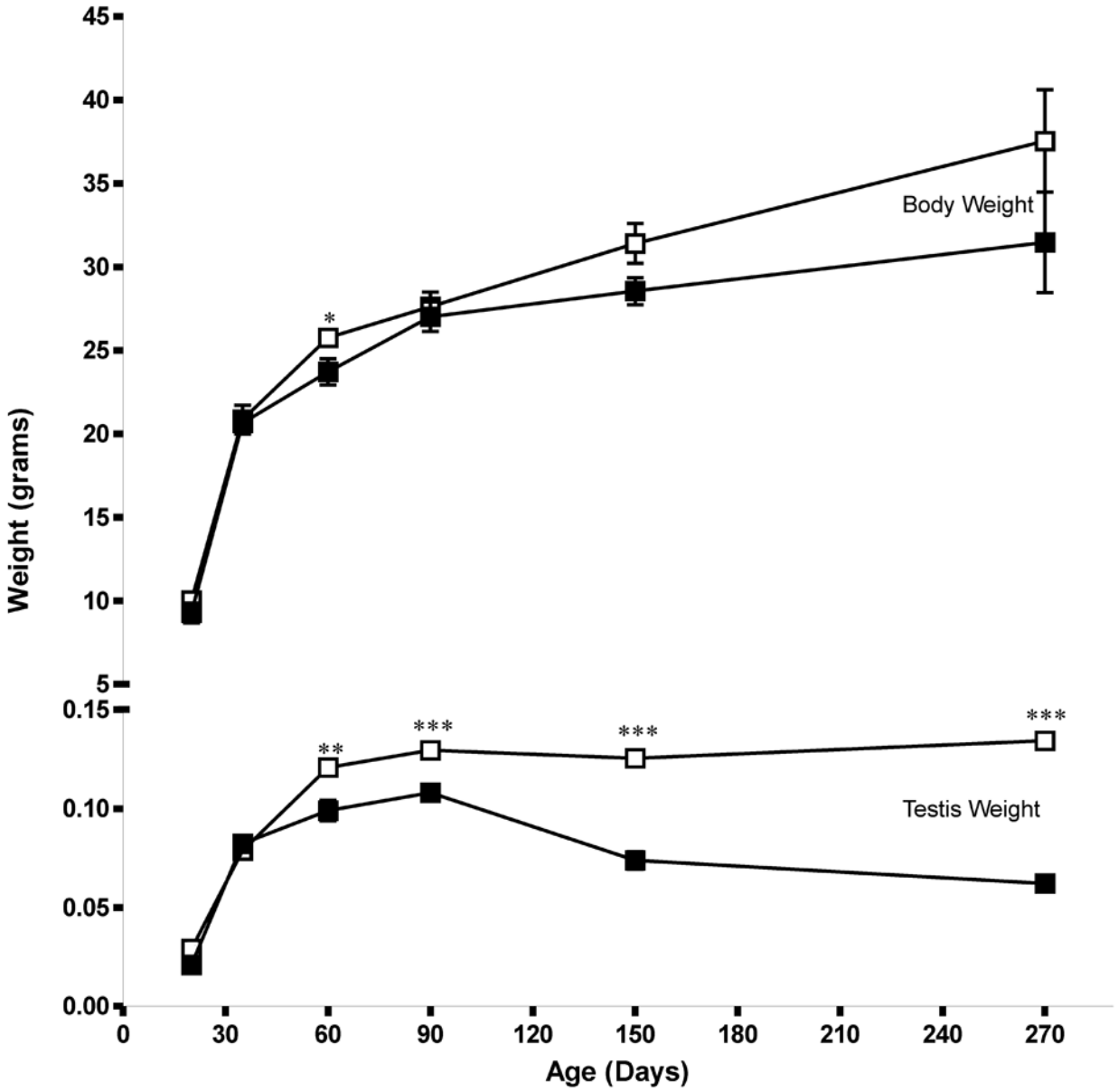

Figure 3.

Sox8$^{-/-}$ animals show an age-dependent effect on testis weight which is not dependent on a reduction in body weight. Testis and body weights were recorded for 4–26 animals per group and plotted +/− SEM. Age in days is indicated along the X-axis. *p<0.05, **p<0.01, ***p<0.001. Open squares, *Sox8*$^{+/-}$,$^{+/-}$ (Sox8 heterozygotes), closed squares, *Sox8*$^{-/-}$ (Sox8 knockouts).

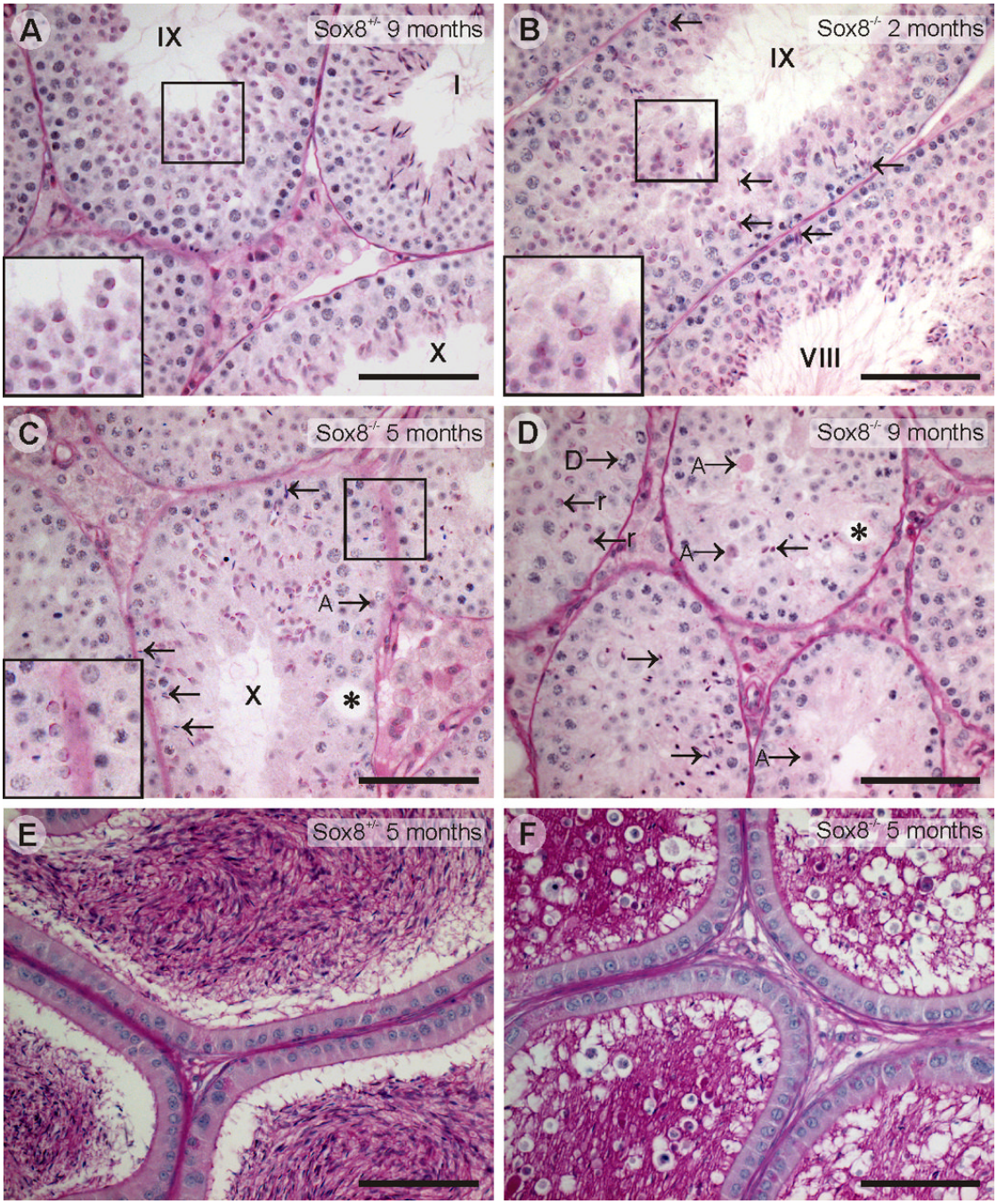

Figure 4.

Testis and epididymal histology of *Sox8*$^{+/-}$ and (Sox8 heterozygotes) and *Sox8*$^{-/-}$ (*Sox8*$^{-/-}$ knockout) animals. A minimum of 3 mice of each age and genotype were examined. **A**. A testis section from a 9-month-old *Sox8*$^{+/-}$ animal showing normal seminiferous tubule histology. The inset illustrates the correct orientation of elongating spermatids in a stage IX tubule ie. with the acrosomes pointing towards the basement membrane. **B**. A 2-month-old *Sox8*$^{-/-}$ testis showing the presence of all germ cell types, but an increased abundance of retained elongated spermatids at stage IX (arrows) ie. spermiation failure. The inset shows the relative disorientation of elongating spermatids at stage IX. Spermiation failure and spermatid disorientation were also visible in stage VIII tubules. **C** A 5-month-old *Sox8*$^{-/-}$ testis showing

a relative reduction in elongating spermatid numbers compared to control animals, increased spermiation failure (arrows) and increased epithelial vacuolation (asterisks) indicative of recent germ cell loss. The inset shows the inappropriate placement of step ~8 round spermatids (normally stage VIII) near the basement membrane of a largely stage X tubule. **D**. A 9-month-old *Sox8*$^{-/-}$ testis showing massive disorganization of the seminiferous epithelium with the presence of elongated spermatids in virtually every tubule, loss of round germ cells, the suggestion of increased apoptosis (A) and the inappropriate placement and timing / juxtaposition of germ cells within the epithelium. **E**. A section of epididymis from a 5-month-old *Sox8*$^{+/-}$ animal showing normal epididymal histology and an abundance of sperm within the lumen. **F**. A section of epididymis from a 5-month-old *Sox8*$^{-/-}$ animal showing the presence of normal epididymal epithelium, but a relative absence of sperm and a massive increase in the number of round germ cells within the lumen. The interstitial tissue of Sox8$^{-/-}$ animals appeared normal at all ages. The age and genotype of animals are indicated in the top right hand corner. The stage of the seminiferous epithelium cycle is indicated in roman numerals. The scale bars represent 100μm. Abbreviations: A – apoptotic cells; As – type A spermatogonia. Asterisk – vacuoles. Arrow – retained elongated spermatids (spermiation failure).

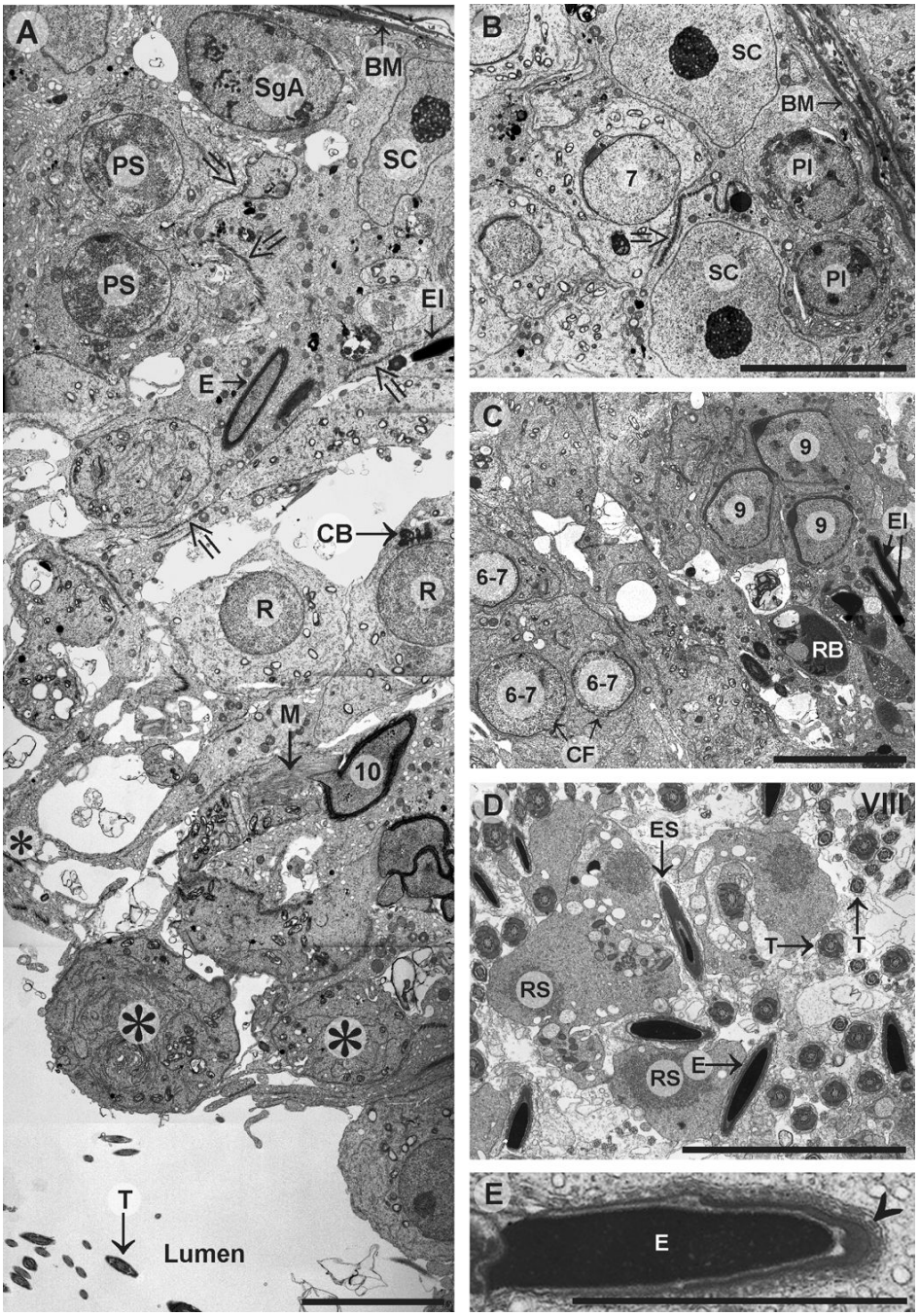

Figure 5.

Electron microscopic examination of *Sox8*$^{-/-}$ (Sox8 knockout) testes. 2 mice of each age were examined. Panels A–C: 5 month old animals; panel D – a 2 month old animal. **A**. Even with advancing age, all stages of sperm development were visible within the seminiferous epithelium, however, as animals aged the placement of germ cells and the synchronization of germ cells development (the cycle of the seminiferous epithelium) became increasingly disorganized. For example, early stage round spermatids (R, step 1–5 as indicated by the lack of acrosome development) were found in the same tubule as elongating (step 10) spermatids and elongating spermatids. The epithelium contained many vacuoles and the Sertoli cell cytoplasm appeared lacey (*), indicative of recent germ cell loss. Inter-Sertoli cell junctions

(the blood-testis barrier, large open arrows) appeared normal. **B**. Shows the inappropriate placement of step 7 round spermatids close to a Sertoli cell nucleus and the blood-testis barrier (open arrows). Sertoli cell nuclei appeared normal. **C**. Illustrates the deregulation of the seminiferous cycle whereby a cluster of step 6–7 round spermatids were seen in the same tubule as a cluster of step 9 elongating spermatids. There was also evidence of failed cytokinesis (CF), or the collapse of cytoplasmic bridges, between sister spermatids. The elongated spermatids were likely the result of a failure of spermiation at step 16 (stage VIII). **D**. A stage VIII tubule from a two month old animal showing a failure of ectoplasmic specialization (ES) resolution which usually occurs at stage VII. Sperm tail cross-sections appeared normal, consistent with the maintenance of sperm motility at least in some sperm. **E**. A retained spermatid showing the presence of actin bundles (the defining feature of the apical ectoplasmic specializations; arrowhead). Abbreviations: BM – basement membrane; CB – chromatoid body; CF – cytokinesis failure; E – elongating spermatid; El – elongated spermatid; M – manchette; PS – pachytene spermatocyte; Pl – preleptotene spermatocyte; R – round spermatid; RB – residual body; RS – aggregations of ribosomes; SC –Sertoli cell; SgA – type A spermatogonia; T – sperm tail. Large open arrows – inter-Sertoli cell junctions / the blood-testis-barrier, Arrowhead- actin bundles. Roman numerals – the stage of spermatogenesis. Arabic numbers – the step of spermiogenesis. Scale bars = 10 μm (A–D) 5μm (E).

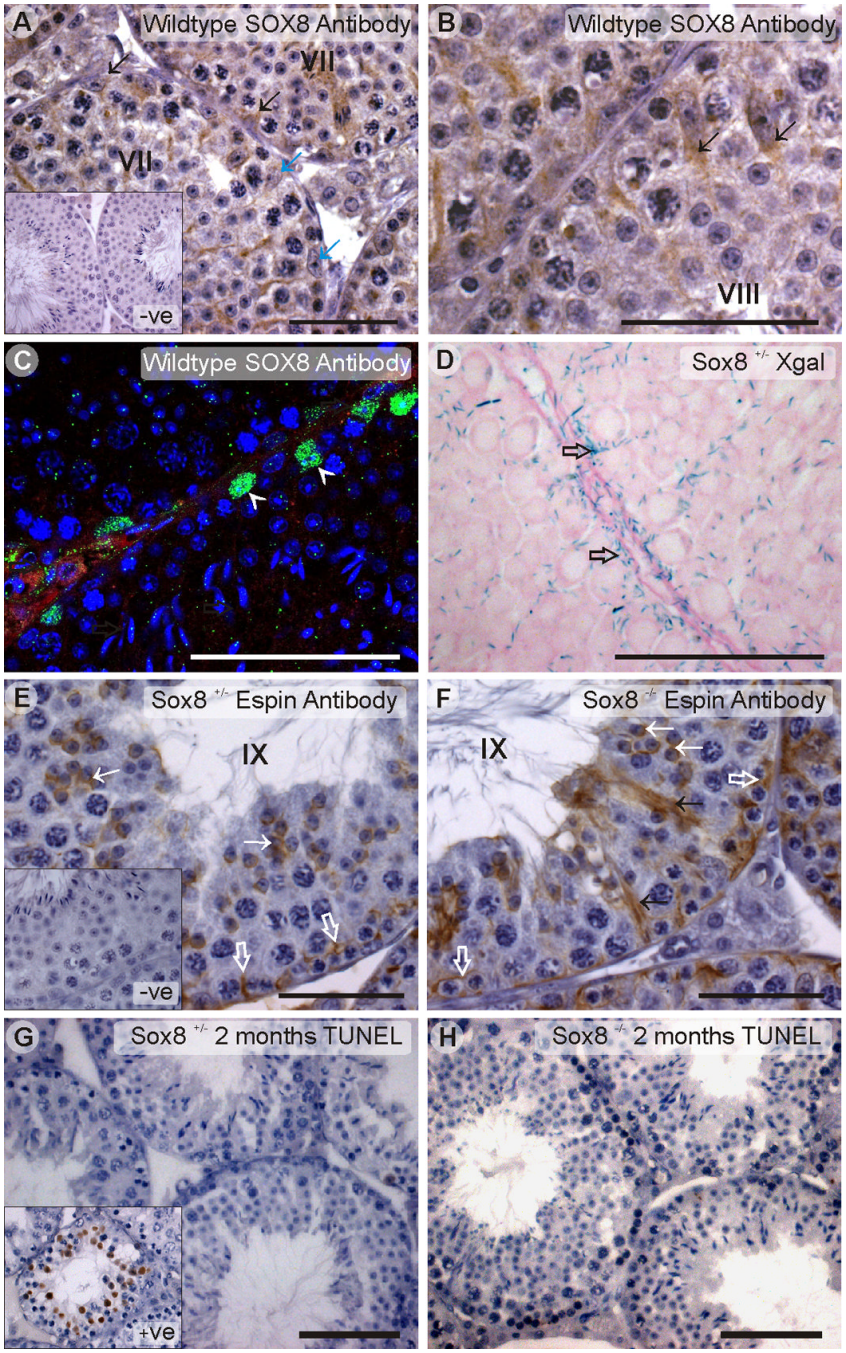

Figure 6.

A. and **B**. Immunohistochemical localization of SOX8 in wild-type testes showing Sertoli cells which were positive for both nuclear and cytoplasmic staining (black arrows) and other which contained only cytoplasmic staining (blue arrows). The inset in panel A shows negative control staining. **C**. Immunofluorescent localization of SOX8 (green) in wild-type testes showing nuclear Sertoli cell staining (white arrowheads). Red staining indicates E-cadherin and blue is DAPI. **D**. LacZ staining of a *Sox8*$^{+/-}$ testis showing the localization of SOX8 specifically to Sertoli cells. Open arrows indicate Sertoli cell cytoplasm. Germ cells showed no evidence of LacZ staining. **E**. and. **F**. The distribution of ectoplasmic specializations, as indicated by Espin immunolocalization, in 2-month-old *Sox8*$^{+/-}$ (E) and *Sox8*$^{-/-}$ (F) animals. White open arrows

indicate the expression of the Sertoli cells derived blood-testis barrier at a level approximately 1 cells depth above the basement membrane ie. separating the spermatogonia and leptotene spermatocytes (basal compartment) from the meiotic and haploid (luminal) compartment. Staining for Espin was also observed around the apical aspect of spermatids from step 8 of spermatid development onwards (white arrows, indicative of the apical ectoplasmic specialization). Black arrows indicate Espin staining on retained elongated spermatids (spermiation failure). The inset on panel E shows a negative control section. **G** and **H**. TUNEL staining to detect apoptotic cells in 2 month old *Sox8*$^{+/-}$ (G) and *Sox8*$^{-/-}$ (H) animals. The inset on panel G shows a positive control section. +ve – positive control (wildtype), −ve – negative control (no antibody). Scale bars A-F= 50 μm, G and H =100 μm. Roman numerals indicate the stage of spermatogenesis. >3 animals were stained and examined.

Figure 7.
The production of SOX8 in the post-natal testis. SOX8 was seen at all ages examined, but relative levels decreased with age consistent with expression by Sertoli cells.

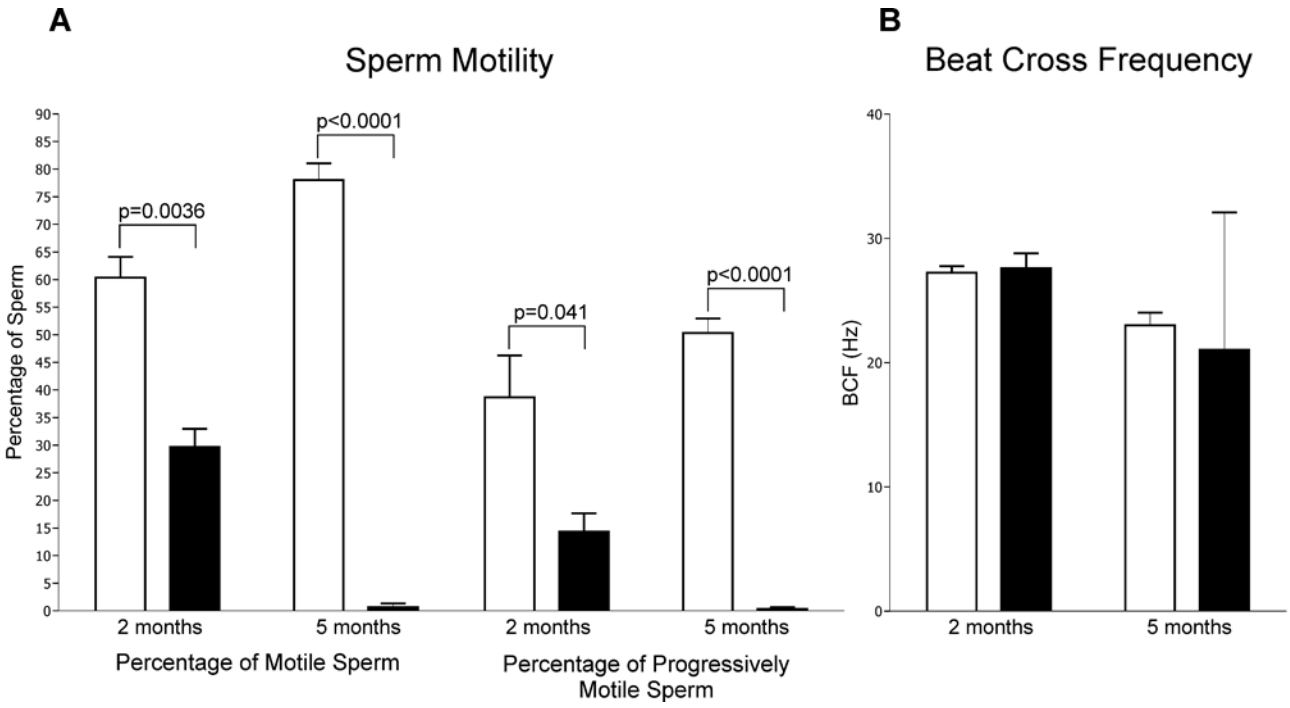

Figure 8.

Motility parameters of caudal epididymal sperm from 2- and 5-month-old *Sox8*$^{+/-}$ (Sox8 heterozygotes) (open bars) and *Sox8*$^{-/-}$ (Sox8 knockout) males (closed bars). **A**. *Sox8*$^{-/-}$ males showed an age-dependent reduction in the percentage of motility sperm produced, and the percentage of progressive motile sperm produced. **B**. In comparison, the beat cross frequency (the frequency with which a sperm track crosses the cell path in either direction), did not change significantly with age.