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Downstream Genes of *Sox8* That Would Affect Adult Male Fertility

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Abstract
Sertoli cells provide nutritional and physical support to germ cells during spermatogenesis. *Sox8* encodes a high mobility group transcription factor closely related to *Sox9* and *Sox10*. Sertoli cells produce SOX8 protein, and its elimination results in an age-dependent deregulation of spermatogenesis resulting in male infertility. This suggests that *Sox8* is a critical regulator of Sertoli cell function for the maintenance of male fertility beyond the first wave of spermatogenesis. To better understand the roles of *Sox8* in testicular development and maintenance of male fertility, we have performed a detailed analysis to explore its downstream genes. We have used mRNA expression profiling to identify affected genes in Sertoli cells in the mutant testes of 2-month-old mice. Expression profiling of the heterozygous and homozygous *Sox8* mutant testes indicates alterations in several important spermatogenesis and blood-testis barrier genes, providing insight into the molecular basis of the defects in *Sox8*$^{-/-}$ testes beyond the first wave of spermatogenesis.

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Sox8 is a member of the SRY-box containing gene family and encodes a high mobility group (HMG) transcription factor that possesses the ability to bind and bend DNA, thus allowing the trans-activation of target genes (Giese et al., 1994; Pontiggia et al., 1994). The Sox family has been divided into 10 subgroups, A–J. Sox8 along with Sox9 and Sox10 compose the SoxE subgroup (Bowles et al., 2000). Sox8 is a product of Sertoli cells and is critical for the maintenance of adult male fertility (O'Bryan et al., 2008). Apart from their obvious supportive role for the seminiferous epithelium, the Sertoli cells harbor clones of differentiating germ cells in cytoplasmic crypts and provide the germ cells with nutrients and growth factors throughout their development (Petersen and Soder, 2006). Sertoli cells regulate the flow of nutrients, growth factors, and mitogens by tight junctions between adjacent cells, creating a structure called blood-testis barrier (Lui and Cheng, 2007). Disruption of Sertoli cell function is suggested to be the cause of pathogenesis in the testicular dysgenesis syndrome (TDS) (Skakkebaek et al., 2001).

Based on its expression pattern in developing testes and ability to induce *Amh* expression in vitro, *Sox8* was initially believed to be a regulator of male sex determination, differentiation, or germ cell development (Schepers et al., 2002, 2003). However, genetic ablation of *Sox8* in

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mice does not result in abnormal sexual development. The lack of defects in sex determination in the mutant mice could be the result of a functional compensation by other SoxE family members (Chaboissier et al., 2004; Koopman, 2005). Detailed analysis revealed that the *Sox8*$^{-/-}$ mice showed decreased adiposity (Sock et al., 2001) and premature osteoblast differentiation, which resulted in poor tarsal development and low bone density (Sock et al., 2001; Schmidt et al., 2005). We independently generated a *Sox8* mutant mouse strain and found that *Sox8*$^{-/-}$ males rarely produced litters, while *Sox8*$^{+/-}$ males and *Sox8*$^{-/-}$ females appeared to be reproductively normal (O'Bryan et al., 2008). In embryonic stages, no overt morphological disruption of cord formation was noted in *Sox8*$^{-/-}$embryos (O'Bryan et al., 2008). Very small numbers of young *Sox8*$^{-/-}$ mice can sire pups with reduced litter size suggesting that at least some sperm resulting from the first few waves of spermatogenesis can be fertile and then they progressively become sterile. Testes weight was normal up to 35 days after birth, but by 2 months of age, the testes of *Sox8*$^{-/-}$ mice were significantly smaller than those of control animals. *Sox8*$^{-/-}$ adult mice (35 days) displayed an obvious infertility phenotype after the first wave of spermatogenesis (O'Bryan et al., 2008). A failure of spermiation was evident in stage IX tubules in 1-month-old mice and was seen more frequently in 2- and 5-month-old mutant animals, and a complete loss of the cycle of the seminiferous epithelium was evident by 9 months. In the gonad, *Sox8* is expressed in Sertoli cells and not in germ cells (Kennedy et al., 2007). SOX8 localization varied within Sertoli cells based on the stage of the spermatogenic cycle. Specifically, within stage I–IX tubules SOX8 protein is localized in nucleo- and cytoplasm, whereas in stage X–XII tubules SOX8 protein was seen only in Sertoli cell cytoplasm (O'Bryan et al., 2008). Sertoli cells usually create 4 to 5 different and ever changing microenvironments concurrently around germ cells. Taken together this suggests that *Sox8* expression in Sertoli cells regulates a set of genes that are essential for male germ cell differentiation and spermatogenesis. Sertoli cells in *Sox8*$^{-/-}$ mice lose this ability in an age-dependent manner, which results in infertility due to the inability to establish full spermatogenesis and shows substantial deregulation of spermatogenesis at 2 months of age.

To gain insight into the molecular basis of this phenotype found in *Sox8*$^{-/-}$ males, we have performed a detailed expression analysis using testes of 2-month-old homozygous and heterozygous mutant mice to identify the downstream genes of *Sox8* that would affect male fertility. By microarray analysis we have determined that loss of *Sox8* affects the expression levels of mRNAs that are important for the functions of Sertoli cells, germ cells, and the blood-testis barrier. The abnormal expression of these genes suggests an involvement of *Sox8*, in part, in the regulation of testicular genes required for male fertility.

Materials and Methods

Mice

The generation of *Sox8* mutant mice has been previously described (O'Bryan et al., 2008). In brief, a 2.6-kb locus of *Sox8* including exons 1 and 2 was replaced with the *lacZ* expression cassette and the floxed *Pgk-neo* cassette. The *Pgk-neo* cassette was removed after germline transmission of the mutation by breeding with *Mox2-Cre* mice. All animal studies were approved by the National Institute of Environmental Health Sciences Animal Care and Use Committee. All experimental data were collected from a minimum of 3 animals of each genotype and stage.

Genotyping

Genotypes of pups were determined by PCR analysis of genomic DNA with primers S8a: 5′-GAG GAC AAA GAT TGG GTC CTG C-3′ and S8b: 5′-GAA GCG TTC GTC TGC TGC C-3′ to detect the wild-type allele (299 bp); and primers S8a: 5′-GAG GAC AAA GAT TGG GTC CTG C-3′ and S8c: 5′-GAT GAA ACG CCG AGT TAA ACG C-3′ to detect the mutant allele (553 bp).

Microarray Analysis

Total RNA was isolated from 2-month-old heterozygous and homozygous mutant mice using TRIzol (Invitrogen). Gene expression analysis was conducted using Agilent Whole Mouse Genome 4×44 multiplex format oligo arrays (014868) (Agilent Technologies) following the Agilent 1-color microarray-based gene expression analysis protocol. Starting with 500 ng of total RNA, Cy3 labeled cRNA was produced according to the manufacturer's protocol. For each sample, 1.65 mg of Cy3 labeled cRNA was fragmented and hybridized for 17 h in a rotating hybridization oven. Slides were washed and then scanned with an Agilent Scanner. Data were obtained using the Agilent Feature Extraction software (v9.5), using the 1-color defaults for all parameters.

The Agilent Feature Extraction Software performed error modeling, adjusting for additive and multiplicative noise. The resulting data were processed using the Rosetta Resolver® system (version 7.0) (Rosetta Biosoftware, Kirkland, WA). In order to identify differentially expressed genes, analysis of variance (ANOVA) was used to determine if there was a statistical difference between the mean heterozygous and homozygous *Sox8* mutants. Specifically, an error-weighted ANOVA with multiple test corrections was performed using Rosetta Resolver. The Benjamini Hochberg False Discovery Rate multiple test correction was used to reduce the number of false positives. Probes with $p < 0.01$ were considered to be differentially expressed.

Quantitative PCR

Heterozygous and homozygous mutant mice were euthanized on postnatal day 15 (P15), P20, P25, P35, and at 2, 3, and 5 months

of age. RNA was isolated from testes using TRIzol (Invitrogen), and cDNA was synthesized using the SuperScript First-Strand Synthesis System (Invitrogen) with random hexamer primers. Quantitative real-time reverse transcriptase PCR (Q-RT-PCR) measurements of individual cDNAs were performed with the ABI Prism 7700 sequence detection system. Gene-specific primers for *Pde4D* (Mm01304777_m1), *Cd164* (Mm01189607_m1), *Cep2* (Mm01329789_m1), *Cldn3* (Mm00515499_s1), and *Cldn23* (Mm00510971_s1) were purchased as pre-designed TaqMan gene-specific probe and primer mixtures (PE Applied Biosystems). The TaqMan rodent glyceraldehyde-3-phosphate dehydrogenase *(Gapdh)* control reagent (PE Applied Biosystems) was used as an internal control. All measurements were performed in triplicate. Values were normalized to *Gapdh* using the $2^{-\Delta\Delta Ct}$ method and expressed as means ± SEM.

Results

Expression Profiling and Validation

Sox8 is critical for the maintenance of male fertility (O'Bryan et al., 2008). Homozygous mutant mice were infertile except for a few that produced small litters of pups while young and then subsequently became infertile (O'Bryan et al., 2008). Weights of testes from $Sox8^{-/-}$ mice were normal at 20 and 35 days after birth, suggesting that alteration of gene expression may not be apparent at this age. The obvious infertility phenotypes were apparent in homozygous mutant mice at 2 months of age; testes were significantly smaller, the seminiferous epithelium showed histological abnormalities, and the number and mobility of sperm were significantly reduced (O'Bryan et al., 2008). Based on these previous observations, we chose 2-month-old testes for gene expression profiling to identify the gene alteration in Sertoli cells.

We performed three independent microarray comparisons of mRNA from testes of heterozygous and homozygous mice at 2 months of age. Statistical analysis identified 1,601 probes as differentially expressed between the homozygous *Sox8* mutant testes and the heterozygous testes (fig. 1A). Of these, 1,314 probes were upregulated, and 108 thereof showed a 2-fold induction or more in the mutant testes (fig. 1B, table 1). 286 probes were downregulated, and 46 of these showed a 2-fold reduction or more in mutant testes at 2 months of age (fig. 1C, table 2). The targeted disruption of the *Sox8* locus resulted in a complete absence of *Sox8* mRNA. Neither the targeted exons (exon 1 and 2) nor the residual coding exon (exon 3) were expressed in the mutant animals (O'Bryan et al., 2008). As expected, *Sox8* was listed as the most reduced gene in the mutant testes by the microarray comparison (26-fold reduction at 2 month of age).

Among the genes identified, some are known regulators of testicular development. These include Sex comb on midleg-like 4 (*Scml4,* 2.1-fold induction); SRY-box containing gene 9 (*Sox9,* 1.4-fold induction); cytochrome P450, family 2, subfamily s, polypeptide 1 (*Cyp2s1,* 3.3-fold induction); DMRT-like family C1a (*Dmrtc1a,* 1.3-fold induction); regulator of sex limited protein 1 (*Rsl1*, 1.3-fold induction); and Anti-Mullerian hormone type 2 receptor (*Amhr2*, 1.5-fold induction). Some of the chromatin remodeling factors known for gene repression were also upregulated in the testes of homozygous mice. These include histone deacetylases 6 (*Hdac6*, 1.3-fold induction) and chromodomain helicase DNA binding protein 6 (*Chd6*, 1.7-fold induction). In addition, the expression of spermatogenesis-associated genes was altered in the mutant testes, including phosphodiesterase 4D (*Pde4D*, 4.5-fold reduction), centrosomal protein 27 (*Cep27*, 3.1-fold reduction), sperm motility kinase 3A (*Smok3a*, 1.2-fold induction), and spermatogenesis associated, serine-rich 1 (*Spats1*, 1.2-fold reduction). Furthermore, there were changes in the expression of genes for proteins perhaps associated with the blood-testis barrier, including occludin (*Ocln*, 1.4-fold induction), claudin 11 (*Cldn11*, 1.4-fold induction), claudin 12 (*Cldn12*, 1.4-fold induction), claudin 10 (*Cldn10*, 1.5-fold reduction), claudin 3 (*Cldn3*, 3.9-fold reduction), and claudin 23 (*Cldn23*, 2.6-fold reduction) (table 1 and 2).

Expression Levels of the Downstream Genes for Sox8 in the Mutant Testes at 2 Months of Age

To validate the microarray results we further analyzed mRNA expression levels of genes by quantitative real-time reverse transcriptase PCR (Q-RT-PCR) in 2-month-old testes. *Cldn23*, *Cep27*, *Cldn3*, and *Pde4D* were chosen because their expression levels were downregulated more than 2-fold in the mutant, and they were known for their involvement in spermatogenesis and formation of the blood-testis barrier. We also included *Cd164* because it encodes a cell surface antigen expressed higher in developing testes than in developing ovaries (McClive et al., 2003) and found it to be highly downregulated (10.2-fold reduction) in the 2-month-old mutant testes (table 2).

Studies of human sperm centrioles showed a greater incidence of centriolar abnormalities in nonprogressively motile spermatozoa when compared with normally motile sperm (Sathananthan, 1994). Males homozygous for a *Sox8* mutation displayed an age-dependent decrease in the percentage of progressively motile sperm (O'Bryan et al., 2008). Together, these data suggest that downregulation of *Cep27* in the mutant testes could be the cause of immotile sperm. Claudins are known as the major com-

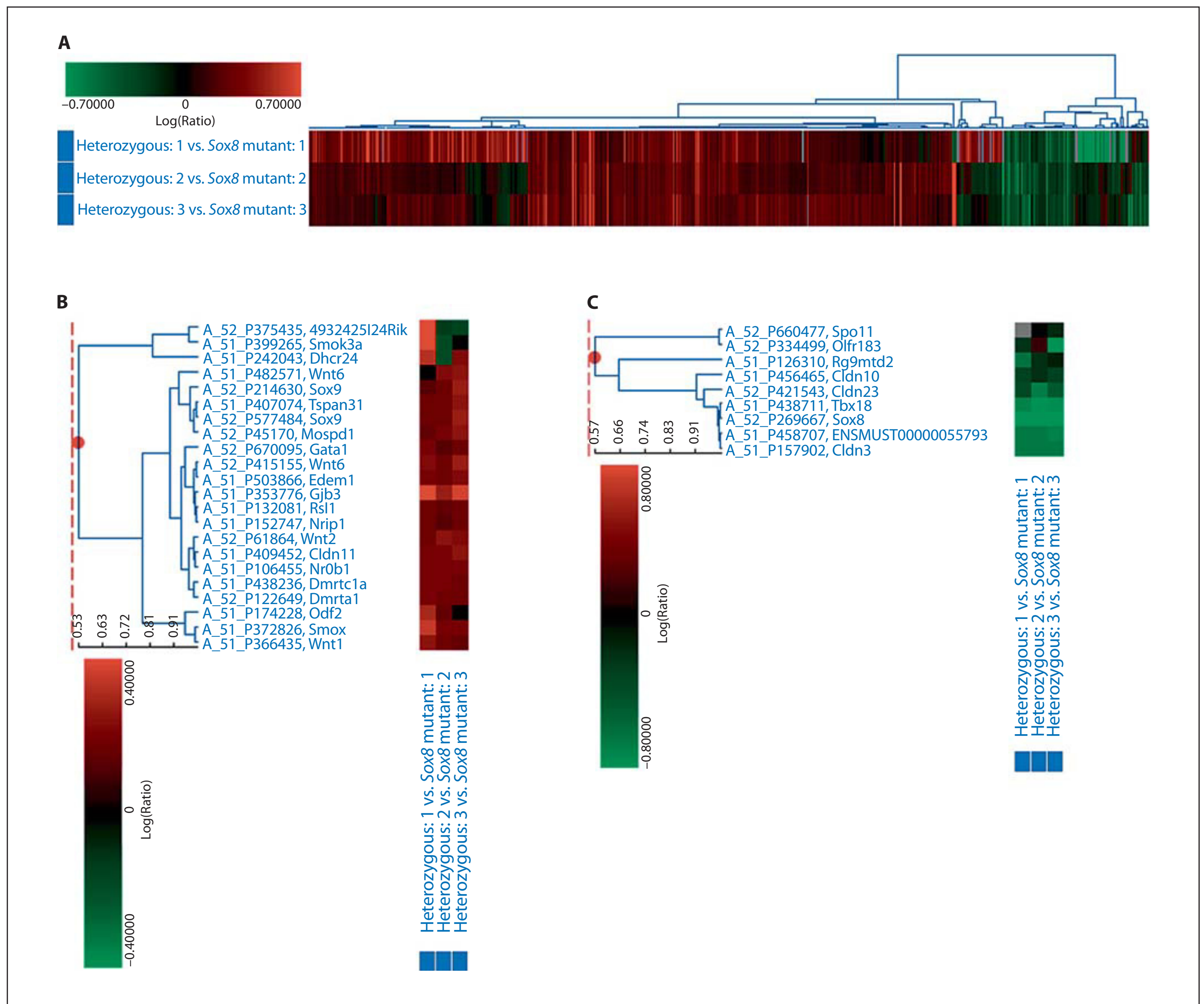


Fig. 1. Microarray comparison of the levels of gene expression in heterozygous and homozygous mutant testes. **A** Induced genes are indicated in shades of red, and repressed genes are indicated in shades of green for the 3 comparisons shown. **B** Representative graph of the upregulated genes in testes of *Sox8*$^{-/-}$ mice. **C** Representative graph of the downregulated genes in testes of *Sox8*$^{-/-}$ mice. Genes listed in **B** and **C** that are known to be involved in testicular development were selected from the results shown in **A**.

ponent of tight junctions in several tissues (Tsukita and Furuse, 2000; Van Itallie and Anderson, 2004). A number of claudins have been shown to be expressed in testes (Lui et al., 2003). Cldn3 was concentrated in the tight junctions near the basal lamina by P20 (Meng et al., 2005). *Pde4D* is expressed in pachytene spermatocytes and during the spermatid elongation phase (Salanova et al., 1999). Based on the localization and function of these repressed genes, we assayed mRNA expression by Q-RT-PCR. As expected, expression levels of all 5 genes were reduced in the mutant testes at 2 months of age (fig. 2A). The expression level of *Cldn23* was reduced most in mutant testes compared to the other 4 genes. *Cep27, Cldn3*, and *Cd164* also showed reduced expression levels in the homozygous mutant testes compared to the heterozygous testes. The change in expression level of *Pde4D* was relatively minor in the homozygous mutant testes at 2 months of age compared to the heterozygous testes.

Table 1. Genes with at least 2-fold increased expression in *Sox8*$^{-/-}$ testes at 2 months of age

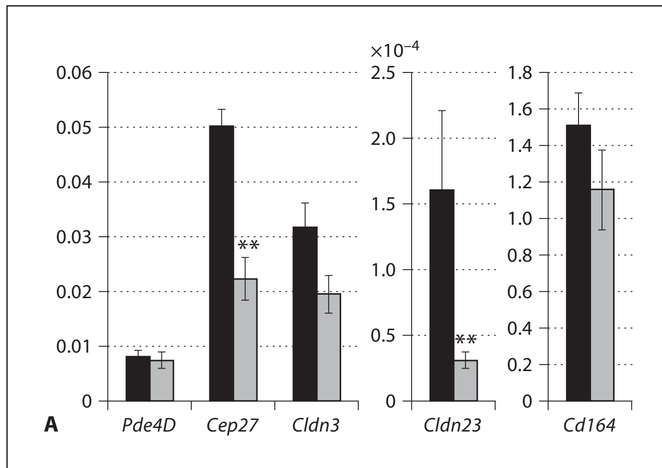
Sequence code	Gene symbol	Sequence description	Fold change	ANOVA p value (p <0.01)
A_51_P370363	*1700030L22Rik*	RIKEN cDNA 1700030L22 gene	77.4	4.2E-14
A_51_P433789	*Agr3*	Anterior gradient homolog 3 *(Xenopus laevis)*	26.3	4.2E-14
A_51_P433796	*Agr3*	Anterior gradient homolog 3 *(Xenopus laevis)*	14.0	4.2E-14
A_52_P731333	*5730526G10Rik*	3 days neonate thymus cDNA, RIKEN full-length enriched library, clone: A630054F14 product: unclassifiable, full insert sequence	10.3	4.2E-14
A_52_P499523	*Scfd1*	Sec1 family domain containing 1	9.3	4.2E-14
A_52_P739568	*Npas3*	0 day neonate cerebellum cDNA, RIKEN full-length enriched library, clone: C230053P15, product: unclassifiable, full insert sequence	7.0	7.0E-06
A_52_P333567	*Adam33*	A disintegrin and metallopeptidase domain 33	6.3	2.0E-10
A_51_P335555	*Snap25*	Synaptosomal-associated protein 25	5.3	7.7E-04
A_51_P464420	*4921508M14Rik*	RIKEN cDNA 4921508M14 gene	5.0	5.1E-04
A_51_P458638	*Spink8*	Serine peptidase inhibitor, Kazal type 8	4.8	4.2E-14
A_51_P180423	*Camp*	Cathelicidin antimicrobial peptide	4.2	4.3E-09
A_51_P514177	*Lrrc2*	Leucine rich repeat containing 2	4.0	8.1E-07
A_52_P420608	*Gm1631*	Gene model 1631NCBI	3.9	4.0E-05
A_52_P188295	*A930021C24Rik*	RIKEN cDNA A930021C24 gene	3.9	5.9E-10
A_51_P131408	*Tnfrsf12a*	Tumor necrosis factor receptor superfamily, member 12a	3.8	1.0E-05
A_51_P452533	*D1Ertd471e*	DNA segment, Chr 1, ERATO Doi 471, expressed	3.8	9.0E-05
A_51_P340456	*Ela3*	Elastase 3, pancreatic	3.7	4.2E-14
A_51_P324633	*Elovl3*	Elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 3	3.6	2.0E-05
A_52_P339959	*Rab3b*	RAB3B, member RAS oncogene family	3.4	4.2E-14
A_52_P298002	*Gch1*	GTP cyclohydrolase 1	3.4	6.0E-05
A_51_P465211	*Wfdc2*	WAP four-disulfide core domain 2	3.3	4.2E-14
A_52_P420357	*Slc15a1*	Solute carrier family 15 (oligopeptide transporter), member 1	3.3	3.3E-03
A_51_P180091	*Cyp2s1*	Cytochrome P450, family 2, subfamily s, polypeptide 1	3.3	4.2E-14
A_51_P302942	*Rasl10a*	RAS-like, family 10, member A	3.3	6.2E-11
A_52_P261184	*Il1rl2*	Interleukin 1 receptor-like 2	3.2	1.0E-05
A_52_P520788	*Scfd1*	Sec1 family domain containing 1	3.2	3.9E-09
A_51_P340699	*Rasl11a*	RAS-like, family 11, member A	3.2	4.8E-09
A_51_P304478	*Tmem28*	Transmembrane protein 28	3.2	5.0E-05
A_51_P153042	*Psg16*	Pregnancy specific glycoprotein 16	3.2	1.5E-11
A_52_P159365	*Sall3*	Sal-like 3 *(Drosophila)*	3.2	5.8E-06
A_52_P21550	*Gcnt1*	Glucosaminyl (N-acetyl) transferase 1, core 2	3.1	9.9E-06
A_51_P385030	*Svs6*	Seminal vesicle secretory protein 6	3.1	4.2E-14
A_52_P452256	*NAP055974-1*	Unknown	3.1	1.1E-03
A_51_P464387	*Hspb8*	Heat shock protein 8	3.0	4.2E-12
A_52_P418952	*Krt79*	Keratin 79	3.0	4.2E-14
A_52_P214347	*Cst11*	Cystatin 11	3.0	1.6E-10
A_51_P138939	*Robo4*	Roundabout homolog 4 *(Drosophila)*	2.9	1.6E-06
A_51_P239203	*Mapk13*	Mitogen activated protein kinase 13	2.9	4.6E-09
A_51_P308048	*Cmtm8*	CKLF-like MARVEL transmembrane domain containing 8	2.8	4.2E-14
A_51_P496905	*Cfi*	Complement component factor i	2.8	1.4E-08
A_52_P180933	*Defb8*	Defensin beta 8	2.7	5.0E-05
A_52_P10622	*Emb*	Embigin	2.7	4.2E-14
A_52_P211418	*6030408C04Rik*	RIKEN cDNA 6030408C04 gene	2.7	1.0E-03
A_51_P465449	*Mybpc3*	Myosin binding protein C, cardiac	2.7	5.0E-07
A_51_P382849	*Emb*	Embigin	2.6	1.6E-12
A_51_P510466	*Pldn*	Pallidin	2.6	4.2E-14
A_52_P964651	*2310033K02Rik*	RIKEN cDNA 2310033K02 gene	2.6	1.0E-02
A_51_P157255	*Sdc2*	Syndecan 2	2.4	4.2E-14
A_51_P362638	*Trf*	Transferrin	2.4	4.2E-14
A_52_P244682	*5430435G22Rik*	RIKEN cDNA 5430435G22 gene	2.4	2.8E-06
A_52_P644297	*Pafah1b2*	Adult male spinal cord cDNA, RIKEN full-length enriched library, clone: A330108B13, product: unclassifiable, full insert sequence	2.4	4.4E-03
A_52_P65237	*Zbtb7c*	Zinc finger and BTB domain containing 7C	2.4	4.2E-14
A_51_P440365	*Frrs1*	Ferric-chelate reductase 1	2.4	2.0E-05
A_51_P100327	*Tap1*	Transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	2.4	3.0E-05
A_52_P407049	*Hoxd10*	Homeo box D10	2.4	1.8E-07
A_51_P267754	*Icam2*	Intercellular adhesion molecule 2	2.4	1.3E-07
A_52_P217796	*1500035H01Rik*	RIKEN cDNA 1500035H01 gene	2.3	3.0E-05

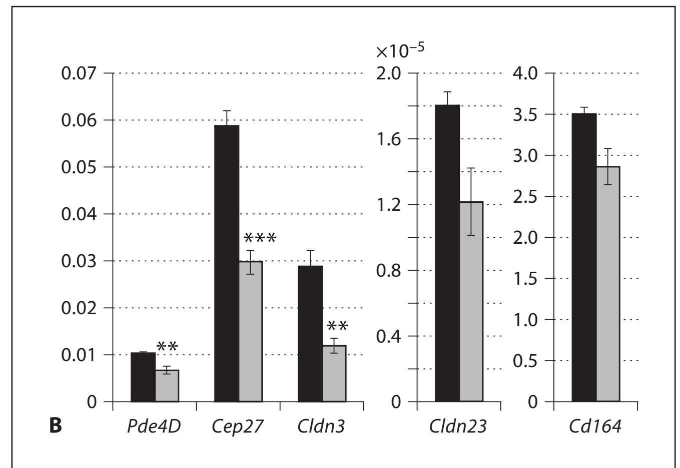
Table 2. Genes with at least 2-fold decreased expression in *Sox8*$^{-/-}$ testes at 2 months of age

Sequence code	Gene symbol	Sequence description	Fold change	ANOVA p value (p <0.01)
A_52_P269667	*Sox8*	SRY-box containing gene 8	–25.65	6.1E-09
A_52_P462657	*TC1060275*	Unknown	–14.25	2.3E-03
A_51_P259848	*Ccdc32*	Coiled-coil domain containing 32	–11.68	1.0E-03
A_52_P421413	*Cd164*	*Mus musculus* adult male thymus cDNA, RIKEN full-length enriched library, clone: 5830453P09, product: CD164 antigen, full insert sequence	–10.20	4.2E-14
A_51_P245324	*4931402G19Rik*	RIKEN cDNA 4931402G19 gene	–7.57	4.2E-14
A_52_P269672	*Sox8*	SRY-box containing gene 8	–5.31	4.2E-14
A_51_P438711	*Tbx18*	T-box18	–4.99	4.3E-08
A_51_P120990	*Pde4d*	Phosphodiesterase 4D, cAMP specific	–4.51	5.3E-03
A_51_P458707	*ENSMUST00000055793*	OLFACTORY RECEPTOR GA_X6K02T2NHDJ-9643949-9642957 [Source: SPTREMBL; Acc: Q7TS05] [ENSMUST00000055793]	–4.07	7.7E-03
A_51_P157902	*Cldn3*	Claudin 3	–3.88	4.2E-14
A_52_P392216	*Dab1*	Disabled homolog 1 *(Drosophila)*	–3.87	4.2E-14
A_52_P529790	*TC1034687*	AK122264 mKIAA0377 protein *(Mus musculus)*, partial (7%) [TC1034687]	–3.74	4.2E-14
A_51_P115178	*Scara3*	Scavenger receptor class A, member 3	–3.70	4.2E-14
A_51_P131494	*Foxk2*	RIKEN cDNA 6230415M23 gene	–3.64	4.0E-05
A_51_P326994	*2810048G17Rik*	RIKEN cDNA 2810048G17 gene	–3.57	6.2E-11
A_51_P497295	*Qpct*	Glutaminyl-peptide cyclotransferase (glutaminyl cyclase)	–3.45	3.6E-13
A_51_P176811	*Tspan10*	Tetraspanin 10	–3.33	3.5E-09
A_52_P1202828	*6620401J10Rik*	RIKEN cDNA 6620401J10 gene	–3.32	9.9E-04
A_52_P1027837	*9330175M20Rik*	RIKEN cDNA 9330175M20 gene	–3.15	5.0E-05
A_52_P246831	*Cep27*	Centrosomal protein 27	–3.10	4.2E-14
A_52_P192085	*Kcng2*	Potassium voltage-gated channel, subfamily G, member 2	–2.94	4.2E-14
A_51_P271200	*Slco1a5*	Solute carrier organic anion transporter family, member 1a5	–2.81	6.8E-10
A_51_P302831	*Dbc1*	Deleted in bladder cancer 1 (human)	–2.77	8.0E-03
A_52_P377838	*AY172399*	Cell-line M2-86 immunoglobulin heavy chain variable region	–2.68	8.5E-03
A_52_P110604	*Hmg20a*	High mobility group 20A	–2.67	3.3E-03
A_52_P421543	*Cldn23*	Claudin 23	–2.56	2.5E-12
A_52_P253044	*Syt13*	Synaptotagmin XIII	–2.47	4.2E-14
A_51_P114094	*Clstn3*	Calsyntenin 3	–2.44	4.2E-14
A_51_P128807	*4930485G23Rik*	MAX gene associated	–2.42	2.6E-12
A_52_P541095	*Unc5a*	Unc-5 homolog A *(C. elegans)*	–2.39	4.2E-14
A_52_P116102	*Tpi1*	Triosephosphate isomerase 1	–2.39	5.0E-09
A_51_P496253	*Slc6a4*	Solute carrier family 6 (neurotransmitter transporter, serotonin), member 4	–2.34	1.2E-04
A_51_P155152	*Ank*	Progressive ankylosis	–2.34	1.1E-03
A_52_P322096	*Patl2*	RIKEN cDNA 4930424G05 gene	–2.31	3.8E-04
A_51_P258894	*Chst2*	Carbohydrate sulfotransferase 2	–2.27	4.7E-04
A_52_P1149183	*AI841723*	Transcribed locus	–2.22	6.0E-03
A_51_P249335	*Sds*	Serine dehydratase	–2.20	1.9E-07
A_52_P185705	*Acn9*	*Mus musculus* adult male corpora quadrigemina cDNA, RIKEN full-length enriched library, clone: B230384K14, product: hypothetical ACN9 containing protein, full insert sequence	–2.15	8.6E-03
A_51_P424739	*Nmnat3*	Nicotinamide nucleotide adenylyltransferase 3	–2.15	3.6E-03
A_52_P304105	*Ppp1r16b*	Protein phosphatase 1, regulatory (inhibitor) subunit 16B	–2.13	4.8E-10
A_52_P723436	*D130062J10Rik*	RIKEN cDNA D130062J10 gene	–2.08	1.3E-03
A_52_P674189	*4930512B01Rik*	RIKEN cDNA 4930512B01 gene	–2.08	9.9E-03
A_52_P67270	*4930515G01Rik*	*Mus musculus* RIKEN cDNA 4930515G01 gene (4930515G01Rik), mRNA	–2.06	6.3E-03
A_52_P168575	*EG434197*	Predicted gene, EG434197	–2.06	2.0E-05
A_52_P599317	*Hs6st2*	Heparan sulfate 6-O-sulfotransferase 2	–2.01	4.2E-14
A_51_P386069	*Rab9b*	RAB9B, member RAS oncogene family	–2.00	4.2E-14

Age-Dependent Expression of the Downstream Target Genes for Sox8 in Mutant Testes

Previously, we have shown that spermiation failure was evident in 1-month-old homozygous mutant mice but becomes more apparent in 2- and 5-month-old homozygous mutant mice, resulting in complete sterility (O'Bryan et al., 2008). This led us to investigate the expression of these selected genes at 3 and 5 months of age. At 3 months, the expression of all 5 genes was reduced in homozygous mutant testes compared to heterozygous testes. The expression lev-





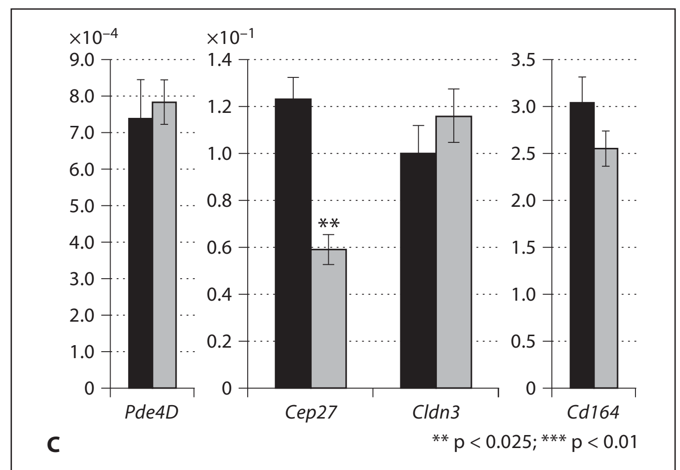
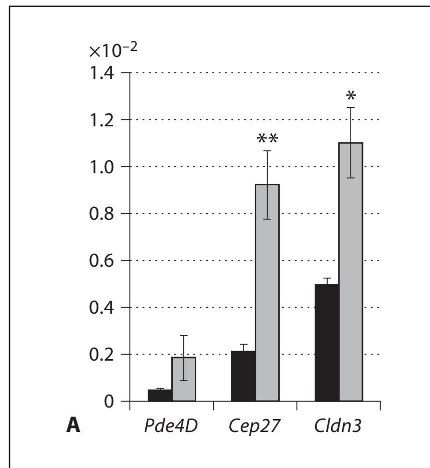


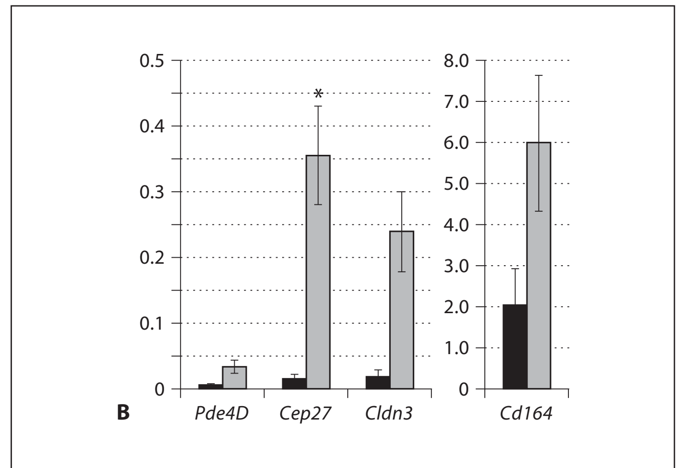
Fig. 2. Temporal pattern of expression of putative downstream genes of *Sox8* in adult testes. Quantitative RT-PCR determined the relative abundance of *Pde4D*, *Cep27*, *Cldn3*, *Cldn23*, and *Cd164* transcripts compared to *Gapdh* mRNA in heterozygous (black bars) and homozygous (gray bars) mutant testes for *Sox8* at 2 months (**A**), 3 months (**B**) and 5 months (**C**) after birth. The data are expressed as mean ± SEM.

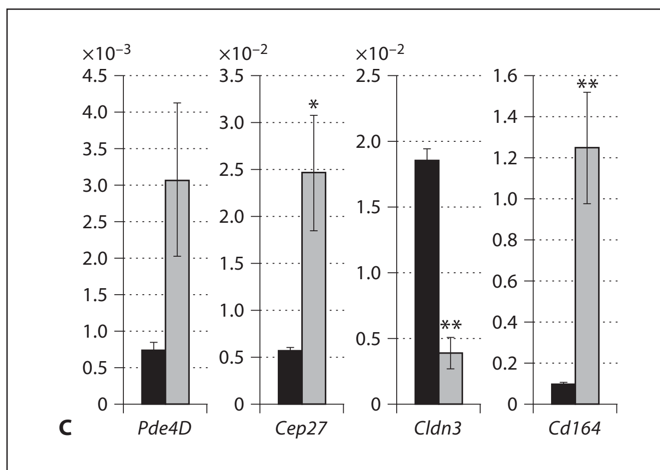
el of *Cldn3* in mutant testes was greatly reduced at 3 months of age relative to the 2-month-old animals (fig. 2B). The level of *Cldn23* expression was higher at 3 months relative to the level in testes of 2-month-old mutants but reduced in comparison to the level in testes of heterozygous animals. The *Cep27* mRNA expression level was reduced at 3 months and 5 months of age in homozygous testes compared to heterozygous testes. In contrast, the expression levels of *Pde4D*, *Cldn3,* and *Cd164* at 5 months of age were unchanged or showed only small changes in homozygous compared to heterozygous testes (fig. 2C), suggesting that these genes are required for male fertility in a particular time frame during spermatogenesis. Expression levels of *Cldn23* in 5-month-old testes were too low to detect.

Sertoli cells proliferate during fetal life until puberty and then cease to divide. In wild type mice, Sertoli cells cease proliferation by about P15 and undergo maturation (Sharpe et al., 2003; Walker, 2003). At the start of puberty (P25) the tight junctions of the barrier are formed (Skinner and Griswold, 1980). Following the establishment of full spermatogenesis at P35 in the mouse (Kramer and Erickson, 1981), each Sertoli cell concurrently supports 4 or 5 different types of germ cells. Our previous study demonstrated that 80% of *Sox8* mutant males were sterile, and ~20% were able to produce small litters at young age (O'Bryan et al., 2008). Where specific expression changes were seen in microarray expression analysis at 2 months of age, we investigated expression at earlier time points, prior to the time that changes in spermatogenesis are observed in *Sox8* mutant testes. To investigate the expression of downstream genes of *Sox8,* RNA was extracted from P15, P20, P25, and P35 heterozygous and homozygous mutant testes.

In contrast to the findings from adult mice, the expression levels of *Pde4D*, *Cep27,* and *Cldn3* were elevated at P15 in the *Sox8* mutant testes before puberty and







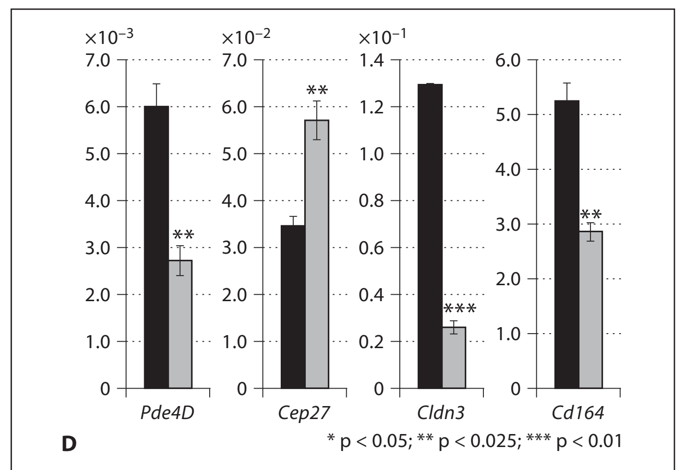


Fig. 3. Effect of *Sox8* mutation on expression of selected downregulated genes before puberty (**A** and **B**, P10 and P20, respectively) at puberty (**C**, P25) and during the establishment of full spermatogenesis (**D**, P35). The relative abundance of *Pde4D*, *Cd164*, *Cep27*, *Cldn3*, and *Cldn23* transcripts in heterozygous (black bars) and homozygous (gray bars) mutant testes compared to *Gapdh* mRNA as an external control is shown (mean ± SEM).

blood-testis-barrier formation (fig. 3A). Similar results were obtained from P20 samples for *Pde4D*, *Cep27*, *Cldn3*, and *Cd164* (fig. 3B). The expression levels of *Pde4D*, *Cep27*, and *Cd164* were increased in $Sox8^{-/-}$ mice at P25, at the time of blood-testis-barrier formation (fig. 3C). At this stage, *Cldn3* expression was decreased in *Sox8* mutant testes (fig. 3C). At the establishment of full spermatogenesis (P35) expression levels of *Pde4D, Cldn3*, and *Cd164*, but not *Cep27* were decreased in the homozygous mutant testes (fig. 3D). These results suggested that the loss of *Sox8* in Sertoli cells might be the cause of differential changes in expression of these genes in an age-dependent manner and in different stages of testicular maturation.

Sequence analysis 2.0 kb upstream of the *Pde4d* and *Cldn23* promoter revealed the presence of a putative SOX binding site (cacacac) between –748 and –742 bp *(Pde4d)* as well as a SOX4 binding site (tctttctctc) between –2095 and –2086 bp and a SOX2 binding site (cacacacaca) between –940 and –931 bp *(Cldn23)* from the initiation codon. Quantitative mRNA expression analysis of the downregulated genes in *Sox8* heterozygous and homozygous mutant testes revealed age-dependent expression changes in the homozygous mutant testes. These data strongly suggest that ablation of *Sox8* results in an age-dependent and within a definite time frame progressive decrease in the expression level of the selected downregulated genes.

Discussion

In the present study we have investigated transcriptional changes in the *Sox8* mutant testis during the stages immediately preceding extensive germ cell loss in the *Sox8*$^{-/-}$mice. Expression profiling identified 108 mRNAs whose expression is significantly (>2-fold) elevated, and 46 mRNAs whose expression is significantly reduced (>2-fold) at 2 months of age, providing a suite of candidate effectors of *Sox8* function for further investigation. We reported earlier that *Sox8* mutant mice displayed an obvious mutant phenotype after the first wave of spermatogenesis in adult mice (35 days), and this became progressively more pronounced in 2-month- and 5-month-old *Sox8* mutant animals (O'Bryan et al., 2008). Some of the genes shown in table 1 and 2 might become apparent due to the block in spermiogenesis or germ cell loss and their effect on whole tissue microarray expression data. Therefore, we chose genes for further analyses based on their expression patterns, i.e., genes specifically expressed in Sertoli cells and repressed more than 2-fold.

Among the genes altered in the *Sox8*$^{-/-}$ testes there are several proteins important for testicular development, spermatogenesis, and formation of tight junctions.

Pde4D is expressed during spermatogenesis in round spermatids and pachytene spermatocytes (Salanova et al., 1999) in addition to Sertoli cells (Levallet et al., 2007). As we reported, in *Sox8* mutant testes spermatogenesis is arrested at stage IX and the formation of round spermatids to elongated spermatids failed (O'Bryan et al., 2008). In *Sox8* mutant testes, expression of *Pde4D* measured by quantitative RT-PCR decreased from 2 to 3 months of age, but then remained unchanged at 5 months of age. This finding suggested that *Pde4D* might be required for spermatogenesis at a specific age. At the same time, it appears that *Sox8* is required for specific timing of *Pde4D* expression. The decrease of the expression of *Pde4D* in the mutant testes may be, at least in part, explained by a reduction of elongated spermatids where *Pde4D* is expressed. However, presence of a SOX binding site strongly suggests that SOX8 might directly regulate the expression of *Pde4D* in Sertoli cells. Further investigation is required to define molecular mechanisms on how SOX8 regulates expression of *Pde4D*.

We have found a number of genes up- and downregulated in *Sox8* mutant testes that are important for tight junctions and the blood-testis barrier, for instance *Cldn3* and *Cldn23* which have a reduced expression between puberty and adulthood. Formation of the blood-testis barrier starts around P11. CLDN3 is first detected at P15 in a diffusive manner and then in the blood-testis barrier at P20 in newly formed tight junctions that regulate the permeability of the barrier as germ cells move from the basal to the adluminal compartment (Meng et al., 2005). Tight junctions in other tissues are shown to regulate the selective transport of ions and nutrients from the blood stream and interstitial fluid (Goodenough, 1999). We also found that *Cldn11* and *Cldn12*, which are crucial for testis tight junction formation (Gow et al., 1999; Bronstein et al., 2000), were upregulated 1.4-fold in the microarray analyses. Lower expression of *Cldn3* and *Cldn23* in the *Sox8* mutant testes suggested that this might be the cause of an altered function of the blood-testis barrier and contributes to male infertility. However, we previously found no overt changes in the expression of Espin and Vinculin (markers for the blood-testis barrier) in *Sox8* mutant testes relative to control testes (O'Bryan et al., 2008). It is possible that the blood-testis barrier structurally forms in the *Sox8* mutant testes, but with an altered function. Thus, further investigation of the blood-testis barrier function in *Sox8* mutant testes is needed.

Spermatogenesis in seminiferous tubules proceeds if an appropriate environment is provided by Sertoli cells (Clermont, 1972). This notion is supported by the study reported here, which demonstrates that the loss of *Sox8* in Sertoli cells downregulates the expression of a group of genes resulting in male infertility. By using microarray analysis and quantitative RT-PCR in a complementary strategy, we have identified expression changes associated with the loss of *Sox8* and consequent loss of spermatogenesis in the mutant mice. Some of these genes are expressed in Sertoli cells and may be direct or indirect targets of SOX8. Further investigation into the function of these genes will help to explain their specific roles in testicular development, male fertility, and its maintenance in an adult age.

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References

Bowles J, Schepers G, Koopman P: Phylogeny of the SOX family of developmental transcription factors based on sequence and structural indicators. Dev Biol 227:239–255 (2000).

Bronstein JM, Chen K, Tiwari-Woodruff S, Kornblum HI: Developmental expression of *OSP/claudin-11*. J Neurosci Res 60:284–290 (2000).

Chaboissier MC, Kobayashi A, Vidal VI, Lutzkendorf S, van de Kant HJ, et al: Functional analysis of Sox8 and Sox9 during sex determination in the mouse. Development 131: 1891–1901 (2004).

Clermont Y: Kinetics of spermatogenesis in mammals: seminiferous epithelium cycle and spermatogonial renewal. Physiol Rev 52: 198–236 (1972).

Giese K, Pagel J, Grosschedl R: Distinct DNA-binding properties of the high mobility group domain of murine and human SRY sex-determining factors. Proc Natl Acad Sci USA 91:3368–3372 (1994).

Goodenough DA: Plugging the leaks. Proc Natl Acad Sci USA 96:319–321 (1999).

Gow A, Southwood CM, Li JS, Pariali M, Riordan GP, et al: CNS myelin and Sertoli cell tight junction strands are absent in *Osp/claudin-11* null mice. Cell 99:649–659 (1999).

Kennedy CL, Koopman P, Mishina Y, O'Bryan MK: Sox8 and Sertoli-cell function. Ann N Y Acad Sci 1120:104–113 (2007).

Koopman P: Sex determination: a tale of two *Sox* genes. Trends Genet 21:367–370 (2005).

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 87:37–45 (1981).

Levallet G, Levallet J, Bouraima-Lelong H, Bonnamy PJ: Expression of the cAMP-phosphodiesterase PDE4D isoforms and age-related changes in follicle-stimulating hormone-stimulated PDE4 activities in immature rat Sertoli cells. Biol Reprod 76:794–803 (2007).

Lui WY, Cheng CY: Regulation of cell junction dynamics by cytokines in the testis: a molecular and biochemical perspective. Cytokine Growth Factor Rev 18:299–311 (2007).

Lui WY, Mruk DD, Lee WM, Cheng CY: Adherens junction dynamics in the testis and spermatogenesis. J Androl 24:1–14 (2003).

McClive PJ, Hurley TM, Sarraj MA, van den Bergen JA, Sinclair AH: Subtractive hybridisation screen identifies sexually dimorphic gene expression in the embryonic mouse gonad. Genesis 37:84–90 (2003).

Meng J, Holdcraft RW, Shima JE, Griswold MD, Braun RE: Androgens regulate the permeability of the blood-testis barrier. Proc Natl Acad Sci USA 102:16696–16700 (2005).

O'Bryan MK, Takada S, Kennedy CL, Scott G, Harada S, et al: Sox8 is a critical regulator of adult Sertoli cell function and male fertility. Dev Biol 316:359–370 (2008).

Petersen C, Soder O: The Sertoli cell – a hormonal target and 'super' nurse for germ cells that determines testicular size. Horm Res 66: 153–161 (2006).

Pontiggia A, Rimini R, Harley VR, Goodfellow PN, Lovell-Badge R, Bianchi ME: Sex-reversing mutations affect the architecture of SRY-DNA complexes. EMBO J 13:6115–6124 (1994).

Salanova M, Chun SY, Iona S, Puri C, Stefanini M, Conti M: Type 4 cyclic adenosine monophosphate-specific phosphodiesterases are expressed in discrete subcellular compartments during rat spermiogenesis. Endocrinology 140:2297–2306 (1999).

Sathananthan AH: Functional competence of abnormal spermatozoa. Baillieres Clin Obstet Gynaecol 8:141–156 (1994).

Schepers GE, Teasdale RD, Koopman P: Twenty pairs of Sox: extent, homology, and nomenclature of the mouse and human Sox transcription factor gene families. Dev Cell 3: 167–170 (2002).

Schepers G, Wilson M, Wilhelm D, Koopman P: SOX8 is expressed during testis differentiation in mice and synergizes with SF1 to activate the *Amh* promoter in vitro. J Biol Chem 278:28101–28108 (2003).

Schmidt K, Schinke T, Haberland M, Priemel M, Schilling AF, et al: The high mobility group transcription factor Sox8 is a negative regulator of osteoblast differentiation. J Cell Biol 168:899–910 (2005).

Sharpe RM, McKinnell C, Kivlin C, Fisher JS: Proliferation and functional maturation of Sertoli cells and their relevance to disorders of testis function in adulthood. Reproduction 125:769–784 (2003).

Skakkebaek NE, Rajpert-De Meyts E, Main KM: Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects. Hum Reprod 16: 972–978 (2001).

Skinner MK, Griswold MD: Sertoli cells synthesize and secrete transferrin-like protein. J Biol Chem 255:9523–9525 (1980).

Sock E, Schmidt K, Hermanns-Borgmeyer I, Bosl MR, Wegner M: Idiopathic weight reduction in mice deficient in the high-mobility-group transcription factor Sox8. Mol Cell Biol 21:6951–6959 (2001).

Tsukita S, Furuse M: The structure and function of claudins, cell adhesion molecules at tight junctions. Ann N Y Acad Sci 915:129–135 (2000).

Van Itallie CM, Anderson JM: The role of claudins in determining paracellular charge selectivity. Proc Am Thorac Soc 1:38–41 (2004).

Walker WH: Molecular mechanisms controlling Sertoli cell proliferation and differentiation. Endocrinology 144:3719–3721 (2003).