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Loss of TSLC1 Causes Male Infertility Due to a Defect at the Spermatid Stage of Spermatogenesis†

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Tumor suppressor of lung cancer 1 (TSLC1), also known as SgIGSF, IGSF4, and SynCAM, is strongly expressed in spermatogenic cells undergoing the early and late phases of spermatogenesis (spermatogonia to zygote spermatocytes and elongating spermatids to spermiation). Using embryonic stem cell technology to generate a null mutation of Tslc1 in mice, we found that Tslc1 null male mice were infertile. Tslc1 null adult testes showed that spermatogenesis had arrested at the spermatid stage, with degenerating and apoptotic spermatids sloughing off into the lumen. In adult mice, Tslc1 null round spermatids showed evidence of normal differentiation (an acrosomal cap and F-actin polarization indistinguishable from that of wild-type spermatids); however, the surviving spermatozoa were immature, malformed, found at very low levels in the epididymis, and rarely motile. Analysis of the first wave of spermatogenesis in Tslc1 null mice showed a delay in maturation by day 22 and degeneration of round spermatids by day 28. Expression profiling of the testes revealed that Tslc1 null mice showed increases in the expression levels of genes involved in apoptosis, adhesion, and the cytoskeleton. Taken together, these data show that Tslc1 is essential for normal spermatogenesis in mice.

The immunoglobulin superfamily (IGSF) is one of four categories of cell adhesion molecules (including the integrins, the selectins, and the cadherins) (21) and consists of cell surface receptors such as neural cell adhesion molecules (NCAMs), intercellular adhesion molecules, and nectins. IGSF members, identified by their characteristic immunoglobulin-like domains, function as adhesion molecules and cell surface recognition molecules involved in various cellular processes, including proliferation, survival, differentiation, and migration (41). Originally known as IGSF4 (immunoglobulin superfamily member 4) (19), TSLC1 has been characterized by several independent research groups, and as a result, this molecule has several names (reviewed in reference 48). IGSF4 was first characterized as a tumor suppressor of non-small-cell lung cancer and termed TSLC1 (tumor suppressor of lung cancer 1) (26, 33). Researchers that found a role for this molecule in adhesion of spermatogenic cells to Sertoli cells termed it a spermatogenic immunoglobulin superfamily member (SgIGSF) (46), and those that revealed a role in driving the synaptic formation of neural cells termed it synaptic cell adhesion molecule (SynCAM) (2). In addition, other names for this molecule include Neel-2 (nectin-like molecule 2) (39) and RA175 (14). Since our interest in IGSF4/TSLC1 is its potential as a lung cancer tumor suppressor gene as well as its involvement in spermatogenesis, we will hereafter refer to the molecule as TSLC1.

TSLC1 is composed of an N-terminal signal sequence and three immunoglobulin-like domains that can interact in a homophilic (28) and heterophilic manner. Only recently has a heterophilic binding partner been identified, namely, class I-restricted T-cell-associated molecule (CRTAM), a receptor primarily expressed on activated cytotoxic lymphocytes (5, 16). TSLC1 also contains a transmembrane domain and a cytoplasmic tail which harbors two important binding motifs, namely, a protein 4.1 binding motif (through which TSLC1 binds the anchoring protein DAL1, which interacts with actin filaments [49]) and a PDZ binding motif (through which TSLC1 binds the PDZ-domain-containing proteins CASK and syntenin [2] and MPP3, a human homologue of the Drosophila tumor suppressor gene Dlg [15]).

Mammalian spermatogenesis involves the differentiation of diploid spermatogonia into spermatocytes and then, through two successive meiotic divisions, into haploid round spermatids. During spermiogenesis, the haploid round spermatids undergo an elongation phase, transforming them into mature spermatozoa. Daughter cells arising from a single spermatogonial stem cell remain connected by cytoplasmic bridges throughout this process, only separating towards the end of spermiogenesis. The process of spermatogenesis is regulated by a variety of hormonal and local factors (9) as well as by direct interaction between spermatogenic cells and Sertoli cells; important structural junctions are formed between the Sertoli cells and spermatogenic
cells at different maturation stages (7). In the testis, TSLc1 is strongly expressed in the spermatogenic cells of the seminiferous tubules; TSLc1 is expressed in spermatogenic cells undergoing the early and late phases of spermatogenesis (spermatogonia to zygote spermatocytes and then elongating spermatids to spermiation), whereas other cell types, including Sertoli cells, lack expression of this protein (47). These findings have led to the hypothesis that TSLc1 functions as a cell adhesion molecule during the early steps of spermatogenesis by binding to a membrane molecule on Sertoli cells in a heterophilic manner.

In view of the spermatogenic phenotype, we have studied Tslc1 null and conditional knockout mice in order to dissect the functions attributed to Tslc1 in spermatogenesis. We show that Tslc1 null male mice are completely infertile. They show a defect in maturation at the spermatid stage of spermatogenesis, characterized by spermatids prematurely degenerating with apoptotic cell death and sloughing off into the lumen, with the production of very few spermatozoa that show severe head malformations and are rarely motile. Thus, Tslc1 is essential for normal spermatogenesis in mice.

MATERIALS AND METHODS

Construction of Tslc1 targeting vector and generation of Tslc1 mutant mice. To generate the Tslc1 targeting vector (pAL1), DNA fragments for the 5′, conditional, and 3′ homology arms were amplified from RPCI-21 PAC -456b9′ DNA by PCR using Platinum PCR High Fidelity supermix (Invitrogen, San Diego, CA) for 10 to 18 cycles. The homology arms consisted of a 4-kb 5′ arm (containing a portion of intron 8 flanked by Ascl sites), a 1-kb conditional arm (containing exon 9 flanked by HindIII sites), and a 4.5-kb 3′ homology arm (containing exon 10 and the 3′ untranslated region flanked by NotI sites), which were cloned into pGEM-T Easy (Promega, Madison, WI), sequenced, and then subcloned into the Ascl, HindIII, and NotI sites of pflexible (45), respectively. A negatively selectable marker, DT-A (50), was then cloned into the Pml site of pflexible. Ten micrograms of AvrII-linearized pAL1 was electroporated into 1 × 106 AB2.2 embryonic stem (ES) cells (from mouse strain 129S5/SvEvBrd) (36) and grown with 3 μg/ml puromycin selection. Cells were cultured on a lethally irradiated SNL7678 feeder layer (30). ES clones were picked into 96-well plates after 9 days of drug selection and expanded, and targeted clones were identified by Southern blot analysis using PCR-amplified probes on Stul- or EcoRv-digested genomic DNA with a 5′ or 3′ conditional probe, respectively, pAl15 is the 540-bp 5′ conditional probe (forward, 5′-GGG GGT TGA AGG CCT AGA AGT GCC CCA CTT TAA ATG A-3′; reverse, 5′-CTT TTC CTT CTC GCA GTC ACT CCT TAA AGC GTG AC-3′), and pAlo10 is the 214-bp 3′ conditional probe (forward, 5′-ACA AAA AAA CTA CCA CTT TTC TCA GAA TTG TGA GGG GCC TAC ACT TT-3′; reverse, 5′-ATT ATT ATT TTT CCA AGT TGT GGG GCC TAC ACT TT-3′). The correctly targeted allele was termed Tslc1Δflexible (m1). To generate null and conditional alleles, a cell line with the m1 allele (EW1) was electroporated with 10 μg of a Cre-expressing plasmid (pTURBO-Cre; GenBank accession no. AF334827) or an Flpe-expressing plasmid (pTURBO+F; GenBank accession no. AF334827) and then selected in G418 (Sigma) and cleaned using MinElute columns (QIAGEN, Valencia, CA). RNAs were treated with DNase I before RT-PCR, and 500 ng total RNA was used for each RT-PCR. Real-time RT-PCR was performed in 96-well plates using a QuantiTect SYBR green RT-PCR kit (QIAGEN) according to the manufacturer's protocols, quantifying the resulting fluorescence using an iCycler system (Bio-Rad, United Kingdom). The RT step was done at 50°C for 30 min, followed by a 95°C activation for 15 s before 50 cycles. The PCR conditions were 95°C for 1 s, 59°C for 1 s, and 72°C for 2 s. The PCR cycle was as follows: 1 cycle at 94°C for 2 min followed by 30 cycles at 94°C for 30 s, 65°C for 1 min, and 72°C for 30 s, with a final cycle of 72°C for 10 min.

Reverse transcription-PCR (RT-PCR). RNAs were extracted from +/+ , c1/c1, and m2/m2 mouse brains (RNAqueous; Ambion, Austin, TX), and 1.5 μg of total RNA was used to generate cDNAs (RetroScript; Ambion). The cDNAs were amplified in 50-μl reaction mixtures using 45 μl Platinum PCR supermix (Invitrogen) and 100 ng of each primer pair with the following PCR cycle profile: 1 cycle at 94°C for 2 min followed by 30 cycles at 94°C for 30 s, 65°C for 1 min, and 72°C for 30 s, with a final cycle of 72°C for 10 min. All PCR products were cloned into pGEM-T Easy and sequenced to confirm their identity. The specific primers used were as follows: for Tslc1 exons 1 to 3, 5′-GAT ATC CAG AAA GAC ACG GCA GTT GAG AGG G-3′ and 5′-CTT CCC GGA TTA GCC CTT GCA GAG GAT A-3′; for exons 5 to 7, 5′-TGA GAG GGT GAG TGG TCG GAC ATG TA-3′ and 5′-CAT ACA GCA TAT AGT CCG AAG CAG CCT T-3′; for exons 9 and 10, 5′-TCC CAG CAG ACG ATG ATG TGT TGT GGT A-3′ and 5′-GAT GAA GAT CTC TTT CTT TTC TGG GGT A-3′ for Tslc1 exons 1 to 3 and 5′-TGC CAG CAC TTG GGT GTA CA-3′ and 5′-CTC ATT GTC GTC GGC TCT CT-3′ were used with the PCR cycle profile shown above.

Quantitative real-time PCR. The expression of Ckfl1, Xmr, and Sty was examined in testes and epididymides from 3-month-old +/+ , +/m2, and m2/m2 mice, with the β-gene used as a control gene (n = 2 for each genotype); the array was performed in triplicate. Total RNA was extracted from RNAqueous (Sigma) and cleaned using MinElute columns (Qiagen, Valencia, CA). RNAs were treated with DNase I before RT-PCR, and 500 ng total RNA was used for each RT-PCR. Real-time RT-PCR was performed in 96-well plates using a QuantiTect SYBR green RT-PCR kit (Qiagen) according to the manufacturer's protocols, quantifying the resulting fluorescence using an iCycler system (Bio-Rad, United Kingdom). The RT step was done at 50°C for 30 min, followed by a 95°C activation for 15 s before 50 cycles. The PCR conditions were 95°C for 1 s, 59°C for 1 s, and 72°C for 2 s. Real-time fluorescence data were captured during the 77°C step of the cycle (the extra step was necessary to avoid interference from primer dimer signals). Melt curve data were obtained to confirm amplification of the correct product in each well. A crossing-point threshold cycle (Cp) value was obtained for each well as the fractional cycle number at which the measured fluorescence crossed the threshold of 60 units, a value chosen such that all reactions in all plates were in log phase. The average Cp was calculated for each gene in each sample. Data were normalized by reference to β-actin, with ∆Cp calculated as follows: ∆Cp = Cp(Tyrosinase) – Cp(β-actin). ∆Cp values were then calculated as the change in ∆Cp for the m2/m2 genotype relative to the wild type. The specific primers used were as follows: for Ckfl1, 5′-TCT CTG GAA ATC GTC CCA CA-3′ and 5′-TCC ACC TAA GAA ATT GGT AGG TTA AAT GTC TT-3′; for Xmr, 5′-TGA GAG GAT CTC TTT CTT TGG GGT A-3′ and 5′-AGA GCA GG-3′ and 5′-TCC ATG TTA CAC TTC TGC CAC-3′; and for Sty, 5′-AGA ATC CAG CTC ATG CT-3′ and 5′-CCA GTC ACC AAC AAAT CAC AC-3′; and for β-actin, 5′-ATG TTA GAG ACC TTC AAC ATC-3′ and 5′-TGC CTT GGA CAA GGT TGG GTT-3′.

Microarray analysis. Microarray analysis was obtained from the testes of 3-month-old +/+ and m2/m2 mice (n = 2 for each genotype). Array hybridizations were performed on the MouseMinGene chip, a DNA microarray representing 11,000 different mouse genes. The clone collection was composed of the NIA 7.4k DNA set (44), 400 hand-picked clones from the NIA 15k mouse cDNA set (22, 42), and ~8,500 hand-selected clones from eight testis cell-specific and subtracted mouse cDNA libraries (8, 29) (Pathology Department, Centre for Microarray Resources, Cambridge, United Kingdom). cDNA labeling and hybridization to arrays were performed as previously described (11). Technical reproducibility was assessed at two levels, namely, within each slide (duplicate of each clone) and between slides (four technical replicates of the experiment). Signal intensities were quantified using BlueFuse software (BlueGnome Limited, Cambridge, United Kingdom), and the data were normalized to the median of overall intensities for the slide. Data from the four technical replicates allowed us to calculate a coefficient of variation for each clone (the quality of the data is inversely related to the coefficient of variation). Data were sorted into 10 equal-width bins based on absolute fluorescence intensities, and

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the most variable clones (the 10% with the highest coefficients of variation) in each bin were excluded from further analysis (43). To identify differentially expressed genes, we used an intensity-dependent Z score. This measures the number of standard deviations a particular data point is from the mean relative to other clones expressed at a similar absolute level (35). Clones with ratios of >2 standard deviations from the mean of the window were considered differentially expressed (95% confidence level). Onto-Express (10, 23) was used to classify the differentially expressed genes according to their gene ontology categories.

Histological analysis. For histology, tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. Tissue sections were stained with either hematoxylin and cosin or periodic acid-Schiff stain and examined by light microscopy. For immunohistochemistry, tissues were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), embedded in paraffin, sectioned, and stained for Tslc1 using either CC2 (a rabbit polyclonal antibody against the C-terminal 18 amino acids of TSLC1 [24]) or EC2 (a rabbit polyclonal antibody against the C-terminal extracellular domain of TSLC1 [amino acids 159 to 223]) [23]). For cryosections, tissues were fixed in 4% paraformaldehyde in PBS, embedded in optimal-cutting-temperature compound, sectioned, stained for actin using Alexa fluor 568-phalloidin (Molecular Probes) or for cell death using an in situ cell death detection kit (Roche Diagnostics, Indianapolis, IN), and imaged by confocal microscopy. Data from terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) counts are based on a total count of 290 tubules per testis. For electron microscopy, tissues were fixed and processed in resin as previously described (1), and transmission electron microscopy was carried out on a Phillips 410LS instrument at 80 kV.

Flow cytometry. Testes were excised from 3-month-old +/- and m2/m2 mice and 6- to 10-week-old 100-μm cell strainers (BD Falcon, Bedford, MA) in PBS before being fixed in 100% ethanol overnight at 4°C. The cells (1.5 x 10^7) were then treated with RNase A (2 μg/ml; Sigma, St. Louis, MO) for 30 min at room temperature and stained with propidium iodide (50 μg/ml; Sigma), and the DNA content was analyzed by flow cytometry. All fluorescence-activated cell sorting data analysis was performed using WinMDI, version 2.8, software.

Fertility studies. To assess male fertility, +/- (n = 6), +/m2 (n = 15), and m2/m2 (n = 15) male mice at 2 to 4 months of age were mated with either +/- or +/- females of different ages at a ratio of 1:1 or 1:2. To assess female fertility, +/- (n = 6), +/m2 (n = 18), and m2/m2 (n = 13) female mice at 2 to 6 months of age were mated with either +/- or +/- males of different ages at a ratio of 2:1. In both cases, the breeding pairs were maintained for a minimum of 2 months or until a litter was born.

Analysis of spermatozoa. Epididymides were removed from four or five (from each group) +/- and m2/m2 male mice at 3 months of age. The cauda region from one epididymis was cut open and incubated in a defined volume of Dulbecco modified Eagle medium with 10% fetal calf serum at 37°C for 30 min to allow dispersion of the contents. A homogeneous suspension was then diluted and spread over a hemacytometer, and the number of mature spermatozoa was determined in duplicate. Serum testosterone levels were quantified for 20 min. Serum testosterone levels were quantified using a Coat-A-Count free testosterone in vitro diagnostic test kit (Diagnostic Products Corporation, Los Angeles, CA) according to the manufacturer’s instructions.

Results. For all comparisons, Student’s t test (unpaired and two-tailed) was used to generate P values.

RESULTS

Generation of Tslc1 null and conditional mice. To assess the physiological role of TSLC1, we have generated null and conditional alleles of the TSLC1 gene by gene targeting. The targeted locus has loxP sites flanking exon 9 and FRT sites flanking the selection cassette (Fig. 1A). Exon 9 encodes the transmembrane domain. We reasoned that the exon 9-deleted form of Tslc1 would not be membrane bound and would lack a functional cytoplasmic domain (splicing of exon 8 with exon 10 would cause a shift in the reading frame). The cytoplasmic domain of TSLC1 (exon 10), containing key protein interaction domains, has been shown to be responsible for its tumor suppressor activities both in vitro and in vivo (27).

The conditional targeting vector was introduced into ES cells, and correctly targeted clones with the genotype Tslc1+/Rdcm1 (+/+ m1) were identified by Southern blotting (Fig. 1A). +/m1 ES cell clones were then exposed to either Flpe (to delete the selection cassette) or Cre (to delete both exon 9 and the selection cassette) to generate ES cell lines with the Tslc1+/Rdcm1 (+/+c1) and Tslc1+/Rdcm2 (+/+m2) alleles, respectively (Fig. 1A). These ES cell clones were used to generate chimeric mice that transmitted the mutated alleles through the germ line. All offspring were genotyped by PCR using the strategy shown in Fig. 1B, which can distinguish between the five possible genotypes (+/+, +/-c1, +/-c1, +/-, and m2/m2).

Interbreeding of heterozygous (+/c1 or +/-m2) mice produced homozygous conditional (+/c1/c1) and null (m2/m2) offspring at the expected Mendelian ratios. These mice were indistinguishable from littermate controls in terms of growth and development and did not show decreased survival or an increased incidence of spontaneous tumor development compared to wild-type mice (by 1 year of age). To confirm that the mutant allele was null, RT-PCR was performed on RNAs extracted from the brains of wild-type and homozygous (m2/m2) mutant mice. Using primers designed against exons 9 and 10, the expected 245-bp product was produced from RNAs from wild-type and c1/c1 mice but not from m2/m2 mice (Fig. 1C and data not shown). Using primers designed against exons 7 to 10, RNAs from wild-type mice produced the expected 291- and 207-bp products (reflecting the alternative isoform of murine Tslc1 that differs by the 84 bp that constitute exon 8), whereas RNAs from m2/m2 mice produced 159- and 75-bp products (Fig. 1C) which, when sequenced, showed splicing of exon 8 (or exon 7) with exon 10. The skipping of exon 9 in the m2 allele results in a reading frame shift which is predicted to result in a nonsense transcript. Immunohistochemistry using anti-Tslc1 antibodies on m2/m2 testis sections showed the absence of any Tslc1 protein, in contrast to wild-type testis sections (Fig. 1D). Thus, we concluded that the Tslc1Rdcm2 allele is null.

Tslc1 is essential for normal male fertility. Adult c1/c1 mice were viable, healthy, and fertile, and thus the c1 allele was not characterized further. However, the conditional inactivation of Tslc1 using c1/c1 mice will make it possible to evaluate the role of Tslc1 in specific tissues.

In contrast, although adult Tslc1 null (m2/m2) mice appeared normal, no offspring ever resulted from matings between 2- to 3-month-old males and wild-type or Tslc1 heterozygous females (Table 1). Older Tslc1 null males (6 to 12 months of age) also showed no evidence of fertility (data not shown), suggesting that a delay in sexual maturation was not the cause of the earlier infertility. A defect in fertility was not observed in Tslc1 heterozygous males (Table 1), and despite Tslc1 expression being detected in oocytes (see Fig. S1A in the supplemental material), Tslc1 null females showed no overt abnormalities in their ovaries (see Fig. S1B in the supplemental material) and possessed fecundity comparable to that of wild-type mice (data not shown). To further investigate the basis for infertility in Tslc1 null males, spermatozoa were isolated from the caudal regions of Tslc1 mice at 3 months of age. As shown in Table 1, Tslc1 null males showed significantly reduced sperm...
FIG. 1. Generation of null and conditional alleles of Tslc1. (A) Tumor suppressor of lung cancer 1 (Tslc1) is a 10-exon gene.

Restriction enzyme sites and fragment sizes are indicated. E, EcoRV; P, PacI; S, StuI. (B) Genotyping of null and conditional tail DNAs was performed by PCR using a combination of two primer pairs (either TSL-5/TSL-3 or TSL-C/TSL-3) which can distinguish between all five genotypes (+/+; c1/c1; +/m2, m2/m2, and m2/m2 -loxP- flank ed exons in Tslc1 null mice. RT-PCR performed on RNAs extracted from wild-type mice showed a 245-bp product corresponding to exons 9 and 10. In contrast, no such product was seen for RNAs from Tslc1 null mice. RNA from wild-type mice showed 291- and 207-bp products corresponding to exons 7 to 10 and 8 to 10 (reflecting the alternative isoform of murine TSLC1 that differs by the 84 bp that constitute exon 8), whereas RNA from Tslc1 null mice produced 159- and 75-bp products, reflecting the deletion of exon 9.

Sperm count compared to their wild-type and heterozygous litters, and the majority of Tslc1 null sperm were static. Thus, Tslc1 is essential for normal spermatogenesis and male fertility. Interestingly, adult Tslc1 null mice did not show a significantly altered level of testosterone (6.3 ± 2.5 mmol/liter; n = 10) compared with their wild-type (9.5 ± 4.2 mmol/liter; n = 9) or Tslc1 heterozygous (8.3 ± 5.6 mmol/liter; n = 4) littersmates; however, this was most likely due to the fact that testosterone is produced by the Leydig cells, which do not express Tslc1 (47) and do not show any abnormalities in Tslc1 null mice.

Loss of Tslc1 causes a maturation defect at the spermatid stage of spermatogenesis. Spermatogenesis is a highly organized series of events, with germ cells progressing through multiple phases of development. Histological analysis of sectioned testes and epididymides from Tslc1 null mice revealed major abnormalities in spermatogenesis. As shown in Fig. 2A, adult testes from wild-type mice at 3 months of age showed spermatogenic cells in all of the different stages of maturation, from spermatogonia to spermatocyte, spermatid, and, finally, spermatozoa, which then passed into the epididymis (Fig. 2C). In contrast, however, adult Tslc1 null testes showed a nearly complete block in maturation at the spermatid stage of spermatogenesis, with round and elongated spermatids degenerating, dying, and sloughing off into the lumen (Fig. 2B). The few surviving elongated spermatids that underwent the next step of maturation to form spermatozoa did not complete the normal maturation process; the heads of the sperm were fatter than those seen in wild-type mice, had abnormal shapes, and sometimes had no or only rudimentary tails. These degenerating spermatids and immature/malformed spermatozoa that sloughed off into the lumen were subsequently passively propelled into the epididymis (Fig. 2D). We also noted that several Tslc1 null testes from mice at 3 months of age showed whole tubules undergoing degeneration, with large vacuoles present (Fig. 2E), and in 5-month-old mice, many tubules showed a complete loss of architecture (Fig. 2F), in contrast to the case in wild-type littersmates (Fig. 2G).

To further characterize the stage of the maturation defect in Tslc1 null testes, we determined the frequency distributions of propidium iodide-stained testicular cells from wild-type and Tslc1 null mice at 3 months of age (Fig. 2H). Compared to wild-type mice, Tslc1 null mice showed a lower HC cell peak (elongated spermatids), an increased 4C peak (most pachytene spermatocytes and a few G1 spermatogonia), and a small increase in the 1C peak (round spermatids). It is possible that failure to complete all stages of maturation may have an effect

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value for mice with indicated genotype</th>
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<tr>
<td>Sperm count (10⁶/cauda)ᵃᵇ</td>
<td>3.9 ± 2.3</td>
</tr>
<tr>
<td>Sperm motility (%)ᵇ</td>
<td>50 ± 11</td>
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<td>Fertility</td>
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ᵃ Sperm count is defined as the number of cells containing a head and a tail.
ᵇ Counts were obtained from four or five mice per genotype. Data are means ± standard deviations.
ᵉ Only two motile sperm (in one of four mice examined) were identified on scanning of the whole slide.
FIG. 2. Analysis of wild-type and Tslcl null testes and epididymides. Hematoxylin- and eosin-stained sections of (A) wild-type testis, (B) Tslcl null testis, (C) wild-type epididymis, (D) Tslcl null epididymis, and (E) Tslcl null testis from mice at 3 months of age are shown. Hematoxylin- and eosin-stained testis sections from (F) Tslcl null and (G) wild-type mice at 5 months of age are also shown. Magnification, ×400 for panels A to D, ×100 for panels E to G, and ×400 for the inset of panel E. Abbreviations: LC, Leydig cells; Sg, spermatogonia; Sc, spermatocytes; St, spermatids (round and elongated); Sz, spermatozoa; iSz, immature spermatozoa; dSt, degenerated spermatids. (H) DNA flow cytometry analysis of testicular cell suspensions from wild-type and Tslcl null (m2/m2) mice at 3 months of age (n = 4 or 5 for each genotype). Arrows highlight the differences between the wild-type and Tslcl null samples. Abbreviations: HC, elongated spermatids; 1C, round spermatids; 2C, spermatogonia; S-ph, spermatogonia synthesizing DNA; 4C, pachytene spermatocytes and G, spermatogonia. (I) Real-time quantitative RT-PCR of three spermatid genes, Cklf1, Xmr, and Ssty, on RNAs extracted from the testes of Tslcl null mice at 3 months of age. The results were normalized to β-actin and shown as relative changes in expression level compared with the wild type. Asterisks indicate statistical significance in the relative changes in transcript levels between the wild-type and m2/m2 samples (P < 0.05).
(via a feedback mechanism) leading to an increase in cell proliferation of spermatogonia (increased 4C) and then to more spermatocytes (increased 4C) and more round spermatids (increased 1C). Taken together, these observations are consistent with the histological findings of a maturation defect at the spermatid stage with an accumulation of spermatids (mostly round spermatids, with some elongated spermatids) and subsequent degeneration and death of the elongated spermatids with sloughing off into the lumen.

To confirm the origin of the sloughed cells, we carried out real-time quantitative RT-PCR on the transcripts for three known spermatid antigens, namely, Cklf1 (11), Xmr (11), and Stsy (8). Both Cklf1 and Xmr were upregulated severalfold in the epididymides of Tslc1 null males; however, Stsy showed a more moderate decrease (Fig. 21). Since the sloughed cells were positive for Cklf1 and Xmr, this indicates a spermatid origin for these cells. Interestingly, the changes in expression levels of these genes in the testis, though lower in magnitude, are the reverse of the situation in the epididymis, with Cklf1 and Xmr being downregulated and Stsy being upregulated. This is consistent with the sloughing of a particular subset of Cklf1\(^{+/+}\) Xmr\(^{+/+}\) Stsy\(^{-/-}\) spermatids into the lumina of the tubules of the testis, with passive propulsion into the epididymis, potentially significantly less (0.080 Xmr matids with sloughing off into the lumen and subsequent degeneration and death of the elongated spermatids (mostly round spermatids). With some elongated spermatids) at the spermatid stage with an accumulation of spermatids (increased 1C). Taken together, these observations are consistent with the sloughing of a particular subset of Cklf1null testes, with passive propulsion into the epididymis, potentially significantly less (0.080 Xmr matids with sloughing off into the lumen.

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**Tscl1 null testes show increased numbers of apoptotic cells.** Adult Tscl1 null testes from mice at 3 months of age weighed significantly less (0.080 ± 0.003 g; n = 25) than testes from their wild-type (0.112 ± 0.004 g; n = 45; P < 0.005) and Tscl1 heterozygous (0.105 ± 0.004 g; n = 27; P < 0.005) littersmates, most likely due to the maturation defect at the spermatid stage of spermatogenesis and the degeneration and death of spermatids with sloughing into the lumina of the seminiferous tubules in these mice. To further examine the nature of the spermatid death, we performed TUNEL analysis (Fig. 3). As shown in Fig. 3A, Tscl1 null testes showed a significantly increased number of TUNEL-positive tubules (tubules containing TUNEL-positive cells; P < 0.01), confirming apoptosis as the major mode of cell death. The number of TUNEL-positive cells per TUNEL-positive tubule was also higher in Tscl1 null testes (Fig. 3B), but this difference was not statistically significant. Interestingly, in contrast with wild-type testes, in which apoptosis occurred in a stage-specific manner. The apoptotic death of cells was also confirmed by electron microscopic examination of the Tscl1 null testes (data not shown).

**Delayed maturation in the first wave of spermatogenesis in juvenile Tscl1 null mice.** In order to unveil the mechanism of maturation arrest of spermatogenesis in Tscl1 null mice, the progression of the first wave of spermatogenesis was examined in wild-type and Tscl1 null (m2/m2) mice (Fig. 4). At postnatal day 7, the histological appearances of the testes from both wild-type and m2/m2 mice were similar, with seminiferous tubules containing only Sertoli cells and spermatogonia. On day 14, when the premeiosis phase of spermatogenesis begins, germ cell differentiation to spermatocytes (primary and secondary) in m2/m2 mice was similar to that seen in wild-type mice. On day 22, the tubules from wild-type and +/m2 mice that had completed the first and second rounds of meiosis showed the presence of spermatids (mostly round spermatids). However, in m2/m2 mice, progression was delayed, and the most advanced germ cells were primarily still in the late pachytene spermatocyte stage (occasional tubules showed the presence of round spermatids). On day 28, the tubules in most wild-type and +/m2 mice had completed meiosis, and numerous round and elongated spermatids could be seen, as well as spermatzoa. However, while m2/m2 tubules showed differentiation of the spermatocytes into round spermatids and some elongated spermatids, there was evidence of spermatids degenerating and sloughing off into the lumen. On day 35, tubules from wild-type mice showed the presence of numerous fully matured spermatzoa. In contrast, although the m2/m2 tubules did show the presence of some spermatzoa, they were immature and malformed, with fat or deformed heads and no or only rudimentary tails. Interestingly, many testes from mice on days 22, 28, and 35 showed the presence of multinucleate giant cells (MNC), containing multiple nuclei within a single cytoplasm, suggesting that one feature of degeneration could be the opening up of cytoplasmic bridges between sister spermatids.

**Tscl1 null testes show some features of differentiation.** We next examined Tscl1-deficient spermatogenic cells for evidence of certain features of differentiation. Staining of spermatids for glycoproteins showed the presence of an acrosome cap in both wild-type and Tscl1 null testes in round and elongated spermatids as well as on immature spermatoozoa-like head structures in the Tscl1 null testes (Fig. 5A and B); this finding was confirmed by electron microscopy (data not shown). Since TSLC1 has been shown to directly associate with the F-actin binding protein DAL-1 (49), and since cytoskeletal elements at the Sertoli cell-spermatid interface play critical roles during spermatogenesis (24, 34), we wanted to examine whether the loss of Tscl1 would result in abnormal actin bundling. However, as shown in Fig. 5C and D, there was no detectable difference in the polarization of F-actin in spermatids from wild-type or Tscl1 null mice.

**Tscl1 null spermatoozoa show major structural abnormalities.** Analysis of the contents of the epididymides from wild-type and Tscl1 heterozygous (+/m2) mice showed mature spermatoozoa with their distinctive nuclear morphology (head region), very little cytoplasm, and a tail (Fig. 6A and B, respectively). In stark contrast, the epididymal contents of Tscl1 null (m2/m2) mice showed degenerating round spermatids with very few spermatoozoa, all of which showed major structural abnormalities, including deformed and irregularly shaped heads, often with a large amount of residual cytoplasm (Fig. 6C to I). Consistent with this, electron microscopy performed on testis sections from wild-type mice showed sperm heads with narrow nuclei and very little surrounding cytoplasm (Fig. 6K). In contrast, sperm heads from m2/m2 mice showed multiple defects, including enlarged nuclei, abnormally shaped nuclei (many of which were degenerated), and large amounts of residual cytoplasm, often with residual body-like changes with dilated mitochondria and vacuoles, suggesting a lack of separation of the residual body from the developing spermatoozoa (Fig. 6L to P). MNC with multiple, often abnormally shaped and enlarged, nuclei within a single cellular cytoplasm were also noted (Fig. 6Q and R).
**Tslc1 null testes show upregulated expression of genes involved in apoptosis, adhesion, and the cytoskeleton.** To understand the molecular consequences of the loss of Tslc1 expression in the testis, we analyzed RNAs from Tslc1 null testes on a germ cell-specific microarray. The data set was normalized and filtered to select differentially expressed genes, with 136 genes being selected as significantly altered in expression (see Materials and Methods). Interestingly, there were many more upregulated genes (103/136) than downregulated genes, which may reflect the accumulation of transcriptionally active round spermatids and the loss of comparatively transcriptionally inactive elongating spermatids and spermatozoa (Fig. 7; see Table S1 in the supplemental material). Differentially expressed genes were then classified according to their likely biological functions, using Onto-Express (10, 23). As shown in Fig. 7, there was an upregulation of a number of proapoptotic genes, again in agreement with the histological findings of increased spermatid apoptosis. There was also an upregulation of several genes with functions related to the cytoskeleton, cell/cell adhesion, and/or maintaining cell junctions. There was no clear functional bias among downregulated genes. When the differentially expressed genes were compared to previous data (11; unpublished data from the same laboratory [E. Clemente, personal communication]) in order to determine their likely sites of expression, it was found that the majority of these genes appeared to be germ cell specific, with only 10 showing expression profiles suggestive of Sertoli cell expression. This may indicate that the majority of downstream effects of Tsc1I loss are germ cell specific or may simply reflect the lower abundance of Sertoli cells in whole testicular tissue. One-third of the upregulated genes were expressed in spermatids, consistent with the histological findings of a maturation defect with an accumulation of spermatids (although this upregulation involved only a subset of spermatid-specific genes), whereas downregulated genes showed no clear bias towards different cell types.

**DISCUSSION**

In multicellular organisms, cells recognize their neighboring cells and adhere to them, forming intercellular junctions that play essential roles in numerous cellular processes (reviewed in reference 20). Furthermore, these intercellular junctions are usually associated with the actin cytoskeleton, which serves to strengthen intercellular adhesion. The seminiferous epithelium of the testis contains two types of intercellular junctions: Sertoli cell-Sertoli cell junctions and Sertoli cell-spermatid junctions (SspJs) (37). Sertoli cells are organized as a single-layered epithelium which cultivates spermatogenic cells throughout spermatogenesis. During the latter half of this process, known as spermiogenesis, spermatids form prominent heterotypic intercellular junctions with Sertoli cells (SspJs), which disappear as the spermatids are released as spermatozoa. These intercellular junctions are formed by cell adhesion molecules on the surfaces of the cells, many of which belong to the IGSF, and a loss of these interactions at these junctions can lead to defects in spermatogenesis. For example, nectin-2 on the surfaces of Sertoli cells interacts with nectin-3 on the surfaces of spermatids, and nectin-2-deficient male mice are infertile due to aberrant morphogenesis and positioning of spermatids and severe spermatozoal malformation (6, 32, 34). Similarly, junctional adhesion molecule C (Jam-C) on the surfaces of spermatids binds to Jam-B on the surfaces of Sertoli cells, and Jam-C-deficient male mice are infertile due to a failure of round spermatids to differentiate into spermatozoa (18).

TSLC1 also known as IGSF4, SgIGSF, SynCAM, and Necl-2) is strongly expressed on the surfaces of spermatogenic cells of the seminiferous tubules, but not on Sertoli cells (47). Thus, TSLC1 has been hypothesized to function as a cell adhesion molecule during the early half of spermatogenesis by forming heterotypic intercellular junctions with a membrane molecule on Sertoli cells. In this study, we have generated a Tslc1 null and a Tslc1 conditional mouse in order to dissect the role of Tslc1 in spermatogenesis. We show that Tslc1 null male mice are infertile due to a maturation defect at the spermatid stage of spermatogenesis. In adult Tslc1 null mice, this defect is histologically characterized by round (and some elongated) spermatids prematurely degenerating and undergoing apoptosis, sloughing off into the lumen, and then appearing in the epididymis. Tslc1 null mice show a delay in spermatogenesis in developing testes by day 22, when the most highly differentiated germ cells in the testes are still at the late pachytene spermatocyte stage, and testes at day 28 show round spermatids (and some elongated spermatids) together with degenerating and dying spermatids that slough off into the lumen, akin to what is seen in adult testes. Apoptosis was shown to be the major mode of spermatid death by histology, electron microscopy, and TUNEL staining. This phenotype is similar in mice deficient in nectin-2 and Jam-C (6, 18, 32, 34); both of these proteins play a role in maintaining SspJs, suggesting that the main feature of Tslc1 loss is a disruption of SspJs.

The histological results were confirmed by flow cytometric analysis of the frequency distributions of propidium iodide-stained testicular cells from adult Tslc1 null mice. This showed a lower percentage of elongated spermatids and a small increase in the percentage of round spermatids. The relative increase in spermatids seen in Tslc1 null mice compared to their wild-type littermates could be due to increased spermatid production, possibly indicating a feedback effect from spermatids regulating spermatogonial proliferation and differentiation. Alternatively, the increase in spermatid number may simply reflect a failure to differentiate further into elongating spermatids.

**FIG. 3.** Tslc1 null testes show increased numbers of apoptotic cells. Tslc1 null (m2/m2) mice show a statistically significant increase in the percentage of TUNEL-positive tubules compared with wild-type (+/+ ) mice (as indicated by the asterisk; P < 0.05) (A) but not in the number of TUNEL-positive cells per TUNEL-positive tubule (B). (C) Detection of apoptosis (TUNEL-positive cells are indicated by arrows) in testis sections from wild-type mice at 3 months of age, with apoptosis occurring in the spermatogonia, in contrast to Tslc1 null mice, which show apoptosis occurring in the spermatids as well as the spermatogonia. Slides for three mice of each genotype were examined, and representative slides are shown. Magnification, ×480.
spermatids, with a consequent accumulation of round spermatids.

During normal spermiogenesis, the round spermatids elongate, condense their nuclei, acquire flagellar and acrosomal structures, and shed a significant amount of their cytoplasm (as residual bodies) to form spermatozoa (7). Defects in these processes lead to a lack of mature spermatozoa (azoospermia), which is a major cause of male infertility in the human population (12). While the round spermatids that were present in the adult Tslc1 null testis showed some hallmarks of normal differentiation, such as the development of an acrosomal cap and normal F-actin polarization, they mostly failed to develop further into elongating spermatids, presumably due to a disruption or loss of normal contact with the Sertoli cell. How-

FIG. 4. Delayed and disrupted first wave of spermatogenesis in Tslc1 null mice. Hematoxylin- and eosin-stained sections of testes from wild-type (+/+) and Tslc1 null (m2/m2) mice at postnatal days 7, 14, 22, 28, and 35 are shown. Magnification for first and second columns, ×200 for day 7 images and ×400 for day 14 to 35 images; magnification for third column, ×1,000. Abbreviations: Sg, spermatogonia; 1° Sp, primary spermatocytes; 2° Sp, secondary spermatocytes; St, spermatids (round and elongated); dSt, degenerated spermatids; Sz, spermatozoa; iSz, immature spermatozoa; MNC, multinucleate giant cells (seen in m2/m2 mice on days 22, 28, and 35; shown at a ×400 magnification).

FIG. 5. Tslc1 null testes show some features of differentiation. Histochemical analysis of testis sections from (A) wild-type and (B) Tslc1 null (m2/m2) mice by periodic acid and Schiff staining showed the presence of acrosomal caps (stained pink) in the spermatids (St) and spermatozoa (Sz). Slides for three mice of each genotype were examined, and representative images are shown. Magnification, ×400. An analysis of testis sections from (C) wild-type and (D) Tslc1 null (m2/m2) mice by phalloidin staining showed normal F-actin (red) polarization in the spermatids of both. Slides for three mice of each genotype were examined, and representative images are shown. Magnification, ×1,600.
ever, histological examination showed that the spermatid defect seen in Tslc1 null mice is not a complete arrest, as a few cells were able to progress to the elongated spermatid stage and then form spermatozoa. Consistent with a defect in spermiogenesis, Tslc1 null spermatozoa are immature and have larger-diameter heads and no or only rudimentary tails. An analysis of the epididymal contents of Tslc1 null mice showed these immature/malformed sperm to be found at only very low levels, and rarely were any motile forms detected. Electron microscopy revealed that they had severe head malformations and abnormal amounts of cytoplasm, often with residual body-like changes, including dilated mitochondria and vacuoles.

Sperm head abnormalities have also been reported for nectin-2 null mice (32).

Interestingly, electron microscopy also showed the presence of multiple malformed sperm head nuclei within a single cellular cytoplasm. These MNC were first seen in the developing testis at day 22. This suggests that one feature of the degeneration is the opening up of the cytoplasmic bridges between sister spermatids. However, multinucleate giant cells have also been seen in the developing testes of testicular orphan nuclear receptor 4 (TR4) null mice (which are infertile), and it was speculated that they may have resulted from spermatocytes with meiosis defects (31). Similarly, multinucleate degenerated...
spermatids were also seen in blind-sterile (bs) mice (40) and Bax null mice (25) and thus may be a nonspecific feature of spermatid degeneration. Alternatively, and more intriguingly, it may be that Tslc1 has a role in maintaining the integrity of spermatid-spermatid interactions as well as the adherens junctions between the Sertoli cells and spermatids (SspJs).

We were able to discover transcriptional correlates for all of the histological, flow cytometry, and electron microscopy findings via microarray and quantitative RT-PCR analysis. Transcription profiling confirmed the expansion in spermatid numbers, with one-third of the upregulated genes being known or putative spermatid transcripts. However, this upregulation did not apply to all spermatid transcripts on the array, suggesting that the increase in spermatid number is due to the expansion of a particular subset of the spermatid population. Functional classification of the deregulated genes showed upregulation of a number of proapoptotic factors and also of genes with putative roles in cytoskeletal function and/or cell adhesion. This reflects the apoptosis and degeneration seen in the expanded spermatid population and the failure of these cells to undergo normal morphological differentiation into elongating spermatids and, subsequently, spermatozoa. Intriguingly, despite the severe failure in the development of late-stage structures such as the sperm tail, there was no corresponding loss of transcripts involved in the production of these structures, suggesting that much of the transcriptional program involved in spermatid differentiation is able to continue despite the failure of the morphological program of differentiation.

Sloughing of degenerating spermatids was confirmed by quantitative RT-PCR, which showed upregulated expression of the spermatid-specific genes Xmr and Cklfl in the epididymis and a consequent slight downregulation of these genes in the testis due to cell loss. Thus, the overall transcriptional picture in the testis is complex, with upregulation of some spermatid genes due to the net expansion in round spermatid numbers but downregulation of other spermatid genes due to cell sloughing. Further analysis of the transcriptional changes (in particular, an in situ examination of transcript localization) will clarify which spermatid stages are retained and expanded in the mutant testis and which are lost through cell sloughing and degeneration. In particular, such analysis should help to reveal which elements of the spermatid differentiation program are innate and which are dependent upon Sertoli cell contact.

Based on the data presented here, we hypothesize that Tslc1 expressed by spermatogenic cells binds to an unknown heterophilic partner on Sertoli cells to mediate a functionally important cell-cell interaction involving transmission of survival and differentiation signals. The loss of Tslc1 does not appear to affect early spermatogenic development (spermatogonia to spermatocytes), possibly because there is no real need for adhesion molecules to maintain close contact between these cell types and the supporting Sertoli cells, as they are already held in place by the latter (due to their physical location within the tubule). It is only when there is a possibility of being shed into the lumen, as occurs at the spermatid stage, that cell adhesion molecules play a critical role in maintaining tight Sertoli cell-spermatid contact. It is in the last steps of spermatogenesis that elongated spermatids are considered to undergo active surface interaction with Sertoli cells in the process of displacement of the spermatid cytoplasm from the condensing nuclei, its casting off as a residual body, and subsequent phagocytosis by the Sertoli cells (13). The major impact of the loss of Tslc1 is in the late stage of spermatogenesis (elongated spermatids to spermatozoa) and underscores this protein’s vital role in cell-cell interactions, particularly between elongated spermatids and Sertoli cells at SspJs. It seems feasible that genetic defects, as well as other factors interfering with normal TSLC1 function in the reproductive system, could lead to male infertility in humans.
REFERENCES


