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The Expression Level of Septin12 Is Critical for Spermiogenesis

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Septins belong to a family of polymerizing GTP-binding proteins that are required for many cellular functions, such as membrane compartmentalization, vesicular trafficking, mitosis, and cytoskeletal remodeling. One family member, septin12, is expressed specifically in the testis. In this study, we found septin12 expressed in multiple subcellular compartments during terminal differentiation of mouse germ cells. In humans, the testicular tissues of men with either hypospermatogenesis or maturation arrest had lower levels of SEPTIN12 transcripts than normal men. In addition, increased numbers of spermatozoa with abnormal head, neck, and tail morphologies lacked SEPT12 immunostaining signals, as compared with normal spermatozoa. To elucidate the role of septin12, we generated 129 embryonic stem cells containing a *septin12* mutant allele with a deletion in the exons that encode the N-terminal GTP-binding domain. Most chimeras derived from the targeted embryonic stem cells were infertile, and the few fertile chimeras only produced offspring with a C57BL/6 background. Semen analysis of the infertile chimeras showed a decreased sperm count, decreased sperm motility, and spermatozoa with defects involving all subcellular compartments. The testicular phenotypes included maturation arrest of germ cells at the spermatid stage, sloughing of round spermatids, and increased apoptosis of germ cells. Electron microscopic examination of spermatozoa showed misshapen nuclei, disorganized mitochondria, and broken acrosomes. Our data indicate that Septin12 expression levels are critical for mammalian spermiogenesis. *(Am J Pathol 2009, 174:1857–1868; DOI: 10.2353/ajpath.2009.080955)*

Septins belong to a highly conserved family of polymerizing GTP binding proteins.[1] They were initially identified in the budding yeast, *Saccharomyces cerevisia*. The septins (Cdc3p, Cdc10p, Cdc11p, Cdc12p, and Shs1p/Sep7p) predominantly localize to the ring(s) that compartmentalize the mother and budding daughter cells. Loss of function for any one of the five septins results in multinuclear and multicellular morphology in the budding yeast.[2,3] In the nematode *Caenorhabditis elegans*, septin (*unc-59* and *unc-61*) mutants generally have normal embryogenesis during early development but multiplex defects in postembryonic development affecting the morphogenesis of the vulva, male tail, sensory neurons and gonad in the larvae.[4] In the fruit fly *Drosophila melanogaster*, septins (*Pnut*, *Sep1*, and *Sep2*) are involved in the formation of ring canals during the incomplete cytokinesis of male and female germ cells.[5] There are at least 13 septin genes in mammalian species, and each septin has multiple splice isoforms. Some septins are expressed ubiquitously, while others are only expressed in well-differentiated cells (eg, neuron or male germ cells).[1] In dividing cells, SEPT2, SEPT6, SEPT7, and SEPT9 have been implicated in the completion of cytokinesis.[6–8] In

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well-differentiated cells, septins are involved in vesicle trafficking and cytoskeleton filament formation.[9–11]

Disruption of septin functions has been implicated in the pathology of many diseases, including cancers, neurodegeneration, and male infertility. *SEPTIN5*, *6*, *9,* and *11* have been observed to be disrupted in reciprocal translocations involving the myeloid/lymphoid or mixed-lineage leukemia gene on chromosome 11 in patients with leukemia.[12] *SEPTIN9* was mapped to a region of loss of heterozygosity at chromosome 17q25.3 in sporadic ovarian and breast cancer.[13,14] SEPT1, 2, and 4 are associated with τ-based helical filaments and contribute to the formation of tangles in Alzheimer's disease.[15] Mutations in *SEPTIN9* cause hereditary neuralgic amotrophy in some families.[16] The roles of septins in mammalian reproduction are just beginning to be revealed. SEPT4 is located at the annulus, a ring-like structure between the midpiece and the tail region of spermatozoa. During spermatogenesis, SEPT4 was found to be essential for the maintenance of proper mitochondrial architecture and establishment of the annulus.[10,11] *Septin4* null mice were viable but sterile in males due to immotile sperm with defective annulus. In addition, the *Septin4* mutant showed defects in the elimination of cytoplasm during sperm maturation.[11] In humans, disorganized annulus/SEPTIN rings were also identified in a subset of human patients with asthenospermia.[10] In a large scale *in silico* research, *Septin12* (Mm.87382) was found to be expressed exclusively in murine spermatogenic cells.[17] We have used microarray analysis to search for genes that are potentially involved in human spermatogenic defects. Of 10 novel testis-specific genes thus identified, one was *SEPTIN12*.[18] In this study, we found lower levels of *SEPTIN12* transcripts in the testicular tissues of infertile men and significant loss of SEPT12 from the sperm samples of men with asthenospermia. We also found reduced SEPT12 expression in spermatozoa with head, neck, or tail defects. To characterize the role of *Septin12* in mammalian spermatogenesis, we knocked out *Septin12* in mouse embryonic stem cells by gene targeting. The chimeric mice showed severe spermatogenic defects. Their reproductive phenotypes include low testis weights, abnormal testicular pathology (hypospermatogenesis or mature arrest of male germ cells), increased apoptosis of germ cells, immotile sperm, sperm with bent or broken tails, and exfoliation of round-headed sperm. In addition, the sperm had acrosomal and mitochondrial defects. Our results indicate that *Septin12* plays critical roles during terminal differentiation of male germ cells in both mice and humans.

Materials and Methods

5′ and 3′ Rapid Amplification of cDNA Ends, Genomic Database Search, and Sequence Alignment

5′ and 3′ Rapid Amplification of cDNA Ends (RACE) reactions were performed using the SMART RACE kit (Clontech, Palo Alto, CA) following the manufacturer's instructions. The open reading frames of the mouse, rat and human Septin12 deduced cDNAs were determined using the open reading frame finder (National Center for Biotechnology Information, NCBI; *http://www.ncbi.nlm.nih.gov/gorf/*). The protein homology motif searching tool Pfam (Sanger; *http://pfam.sanger.ac.uk/*) was used to analyze the mouse and human names of the conserved domains and to display the alignment. Alignment of genes of different species was performed using the Megalign program of the DNASTAR software package (DNASTAR, Inc., Madison, WI). Alignment of all Septin12 genes and proteins of different species was performed by using the ClustalW (EMBNet; *http://vit-embnet.unil.ch/software/ClustalW.html*) and BoxShade 3.21(EMBNet; *http://vit-embnet.unil.ch/software/BOX_form.html*).

Reverse Transcription-PCR for Expression Patterns of Septin12

Human Total RNA Panel (Clontech, Palo Alto, CA) and different organs from adult mice (day 80) were used to study the expression pattern of *Septin12.* To study the expression profiling in different developmental stages of spermatogenesis, testicular tissue was retrieved from mouse of postnatal days 1, 5, 10, 15, 20, and 35, and from adult (>day 80) mice. The germ cell population within the seminiferous tubules mainly consists of spermatogonia while spermatocytes are absent in the mouse testes of postnatal day 1 and day 5. In days 10, 15, 20, 35, and 80, spermatogonia develop into primary spermatocytes, pachytene spermatocytes, round spermatids, elongating spermatids, and mature spermatozoa, respectively.[19,20] The *c-kit* knockout mice were purchased from Jackson Laboratory (Bar Harbor, ME). Total RNA was extracted from human testis, various mouse organs, and different developmental stages of mouse testes. The PCR conditions and product detection were performed as described in our previous publication.[21]

Separation of the Testicular Germ Cell Populations and Sperm Preparation

Separation of spermatogenic cells was performed based on the density of different types of germ cells by a centrifugal system, as described previously.[22] After de-capsulation and enzyme digestion, germ cells suspensions were filtered through 35 μmol/L nylon filters (Falcon; Becton Dickinson, Franklin Lakes, NJ), followed by centrifugation using a Kubota centrifuge 3330 (Kubota Corp., Tokyo, Japan). Germ cells of different developmental stages were collected. Mature spermatozoa were collected from the cauda epididymis of adult male mice. Finally, suspensions were centrifuged with maximal force for 10 minutes and were spread on a slide and air-dried.

Generation of Anti-SEPT12 Antibody, Western Blot Analysis, and Immunofluorescence Study

A peptide (TMEEREAFRRRIQQNLRT, amino acids 200 to 217 in NP 653206) of SEPT12 was used to immunize rabbits (Protech, Taiwan) according the methods described in our previous study.[23] Western blot analysis for SEPT12 was performed according to the standard protocols with minor modifications.[21] For immunofluorescence assay (IFA), the slides were treated with 0.1% Triton X-100, washed twice with Tris-buffered-saline, followed by incubation with the anti-SEPT12 antibody (1:100) for 60 minutes at room temperature. Following the washing steps with Tris-buffered-saline, sections were exposed to goat anti-rabbit Alexa Fluor 488 (Molecular Probes, Carlsbad, CA) for 60 minutes at room temperature and washed with Tris-buffered-saline. Lectin peanut agglutinin-conjugated with Alexa Fluor 568 (10 mg/ml) (Molecular Probes, Carlsbad, CA) was used to locate the acrosomes in testes and spermatozoa. The midpiece of spermatozoa was staining by the mitochondrial tracker (Molecular Probes, Carlsbad, CA). To stain the nuclei, 4,6-diamidino-2-phenylindole was used.

Human Samples

The study was approved by the Institutional Review Board of National Cheng Kung University Medical Centre. All patients who underwent diagnostic testicular biopsy or sperm retrieval or semen analysis agreed to provide spare samples for further study. Informed consent was obtained from all patients enrolled in the study. For real-time reverse transcription (RT)-PCR analysis of the testicular tissue, eight patients with obstructive azoospermia and normal spermatogenesis and 16 patients with non-obstructive azoospermia were recruited. All patients underwent diagnostic testicular biopsy or sperm retrieval. Some testicular samples were immersed in Bouin's solution and sent for histopathological diagnosis. The testicular histopathology was confirmed by two specialists, and was categorized according to the most advanced pattern of spermatogenesis present. The remaining tissues were cryopreserved for RNA extraction. Of the 16 patients with non-obstructive azoospermia, 13 were diagnosed with hypospermatogenesis and three were diagnosed with maturation arrest at the spermatid stage. Real-time RT-PCR analysis of the *SEPTIN12* transcript was performed as described previously.[24] For the IFA, semen samples were collected from 19 men with normal semen parameters and 11 infertile men with asthenozoospermia. The ejaculate was obtained by masturbation after a minimum 48 hours of sexual abstinence. The assessment of sperm concentration was performed as recommended by the World Health Organization's recommendations using a modified Neubauer chamber and positive displacement pipettes for proper dilution of the ejaculate.[25]

Targeting Vector and Generation of Mutant Mice

The animal study was approved by the Institutional Review Boards of two institutes: National Cheng Kung University Medical Centre and National Taiwan University Medical Centre. A genomic fragment carrying the entire mouse *Septin12* locus was constructed in bMQ-374d12 BAC clone (Sanger institute, UK). The genomic fragment of *Septin12* constructed in pL253 was used to replace the wild-type allele of *Septin12* in 129Sv mouse embryonic stem cells (MESCs). MESC clones containing the targeted allele were identified by Southern blot analysis. Several clones were isolated and transfected with a vector encoding the Cre recombinase to delete a 2.58 kb region of *Septin12* locus (spanning from exon3 to exon7). Clones with the *Septin12* knockout allele were

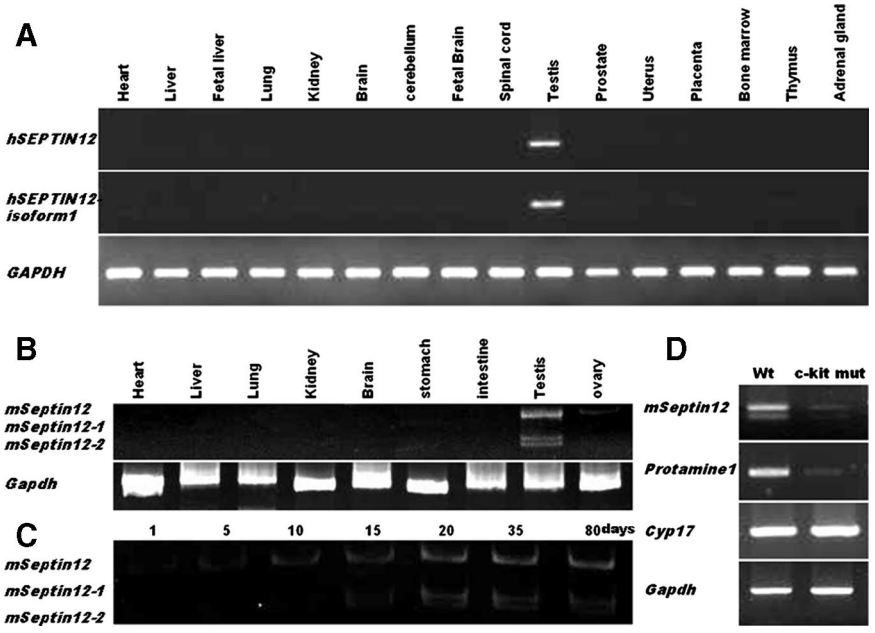

Figure 1. RT-PCR expression profiles of *Septin12* in humans and mice. The expression of *hSEPTIN1, hSEPTIN12-1, mSeptin12, mSeptin12-1,* and *mSeptin12-2* is restricted to the testis in humans (**A**) and mice (**B**). *GAPDH* was used as a loading control. **C:** *Septin12* expression in the mouse testes of different postnatal days. **D:** Total RNA from testes of wild-type (WT) mice and *c-kit* mutant mice, which lacked germ cells, was amplified by RT-PCR. *Protamine1* was a male germ cell maker. *Cyp17* is a Leydig cell marker.

identified by Southern blot analysis and were injected into C57BL/6J blastocysts.[26] The testicular tissues of chimeric mice were genotyped to determine the ratio of mutant and wild-type alleles using strain-specific micro satellite markers.[27]

Semen Analysis and Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling Assay

Spermatozoa collected from the caudae epididymides of 20-week or older *Septin12* chimeric mice were suspended in human tubal fluid medium (Irvine Scientific, Santa Ana, CA). For sperm counts, sperm were immobilized by dilution in water and counted with a hemacytometer in duplicates. For determining percent motility, the sperm medium was diluted to 10^6/ml with human tubal fluid and spotted onto a glass slide. A total of 200 sperm (both motile and immotile) were counted under a microscope in duplicates to obtain an average percent motility. For the determination of apoptotic cells, an apo-bromodeoxyuridine terminal deoxynucleotidyl transferase dUTP nick-end labeling assay (Invitrogen, Carlsbad, CA) was preformed. Testes were fixed in Bouin solution and cut into 5-μm thick paraffin sections. The DNA break sites of testis sections were labeled by the Apo-bromodeoxyuridine kit following the manufacturer's protocol.

Electron Microscopy

About 10^6 mouse spermatozoa were washed in 0.1 M/L phosphate buffer (pH 7.2) and prefixed in the same buffer with 2% paraformaldehyde and 1.25% glutaldehyde at 4°C for 10 minutes. The scraped fixed cells were concentrated by centrifugation for 3 minutes at 2000 $\times$ *g*. Pellets were postfixed in 1% OsO_4 aqueous solution at room temperature for 1 hour. They were washed with ddH_2O for 10 minutes three times, and were dehydrated in increasingly grade ethanol and pure propylene oxide. The samples were embedded in Epon at room temperature and polymerized in oven at 55 C for 1 day, and 80-nm thin sections were cut from the blocks and collected onto the grids. Sections were counterstained with lead citrate and uranyl acetate and observed with a JOEL 1200 transmission electron microscope (JOEL Institute Inc., Lexington, MA).

Results

Septin12 *Is Expressed in the Postmeiotic Germ Cells*

To illustrate the genomic structure of *Septin12* in different mammalian species, RACE analysis was applied to mouse, rat, and human testicular RNAs. Three, one, and

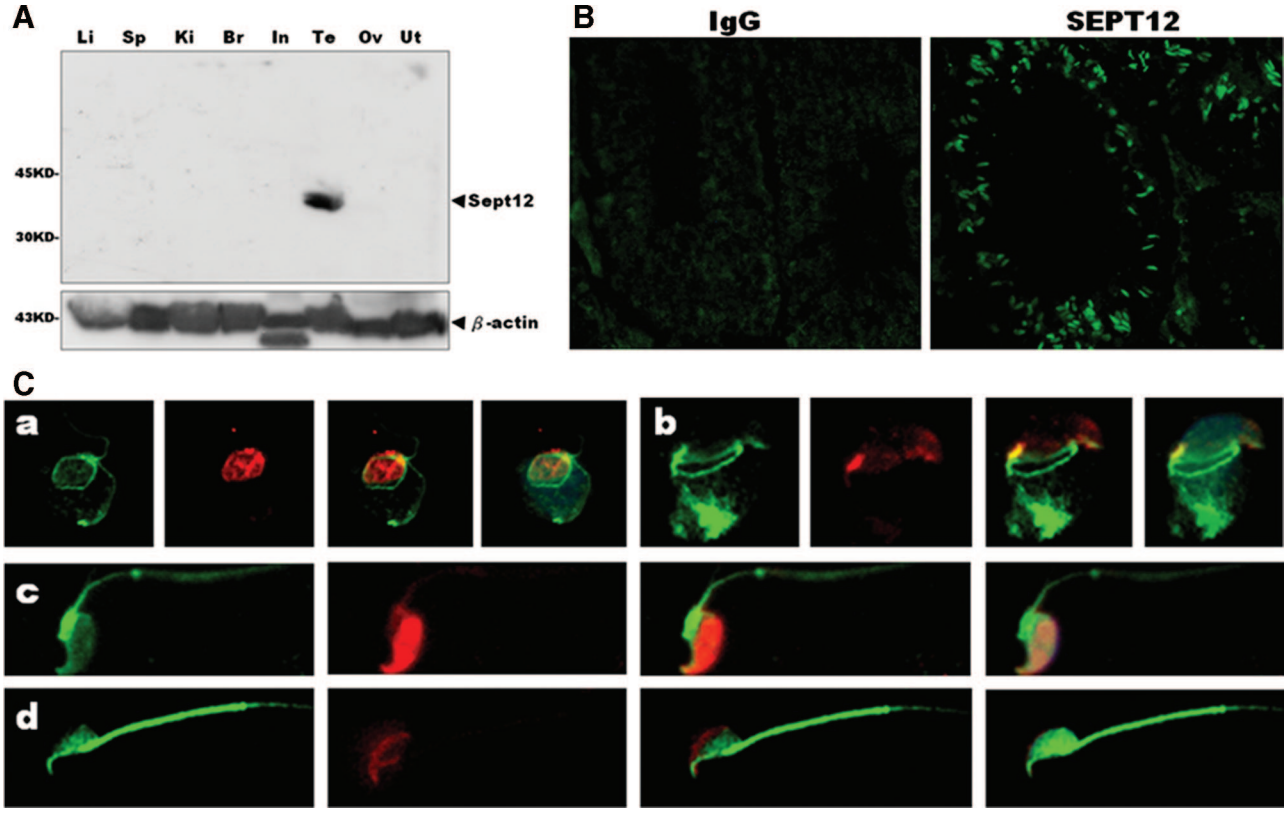

Figure 2. Expression pattern and localization of the SEPT12 protein. **A:** Western blot analysis of SEPT12 in multiple mouse tissues, including liver (Li), spleen (Sp), kidney (Ki), brain (Br), intestine (In), heart (He), testis (Te), ovary (Ov), and uterus (Ut). β-actin was used as a loading control. **B:** Immunofluorescence detection of SEPT12 (green) on testicular sections of the adult mouse. **Left panel**: preimmune antiserum, **Right panel**: anti-SEPT12 antibody. **C:** Immunofluorescence microscopy showed multiple localizations of SEPT12 signals. From left to right: SEPT12 signal (green); acrosome marker (red); merge of SEPT12 and acrosome signals; merge of SEPT12, acrosome and nuclear DNA (blue) at step 7 (**a**), steps 10 to 11 (**b**), steps 14 to 16 (**c**) of spermiogenesis and mature sperm (**d**). In mature sperm, SEPT12 signals are located at the head, neck and midpiece with scanty signals at the tail. Scale bar = 50 μm.

two transcriptional isoforms were identified in the mouse, rat, and human, respectively. *Septin12* contains 10 exons in all three species and the amino acids sequences of SEPT12 are highly conserved in these three species (see supplemental Figure S1 at *http://ajp.amjpathol.org./*). RT-PCR was performed to determine the expression patterns of *Septin12* in multiplex tissues in humans and mice. The different isoforms are mainly expressed in the testis (Figure 1A and B). The full length transcript is also slightly expressed in the mouse ovary (Figure 1B). The full-length transcript started to appear in the testes of 5-day old mice, a developmental stage equivalent to mitotic phase of spermatogonia. Two other isoforms of *Septin12* started to be transcribed at day 15, corresponding to the stage of pachytene spermatocytes (Figure 1C). The transcript level decreased in the testicular tissue of *c-kit* mutant mice, which are deficient in germ cells (Figure 1D). These findings suggest that *Septin12* starts to be expressed in the meiotic stage in the male germ line. Western blot analysis using polyclonal antibody showed SEPT12 is only expressed in the mouse testis (Figure 2A). Immunofluorescence staining using mouse testicular sections showed the signals are confined to postmeitoic germ cells in the seminiferous tubules (Figure 2B, right panel). *In vitro*, SEPT12 recombinant protein also forms filament-like structure in 293Tcells (see supplemental Figure S2 at *http://ajp.amjpathol.org./*), a finding consistent with a previous study using Chinese hamster ovary cells.[28] Using IFA on mouse germ cells isolated from the mouse testis, we found the twisted filament-like structures of SEPT12 started to appear around the acrosome at step 7 of spermiogenesis (Figure 2, C and a). At steps 10 to 11 of spermiogenesis, SEPT12 forms a circular structure between the edge of acrosome and the perinuclear mantle of the manchette (Figure 2, C and b). With the formation of mitochondria, SEPT12 starts to localize at the neck and annulus regions. The SEPT12 filaments also cover the mitochondrial area (Figure 2, C and c). In mature spermatozoa, the SEPT12 signal is located at the sperm head, neck, and midpiece with scanty signals at the tail (Figure 2, C and d). The results of IFA using pre-immune serum as a negative control are shown in supplemental Figure S3 at *http://ajp.amjpathol.org./*. The finding suggests SEPT12 is a structural protein involved in the formation of the subcellular compartments, head, neck, midpiece, and tail, during terminal differentiation of germ cells.

Expression Patterns of SEPT12 in Human

We asked whether loss of *SEPTIN12* was involved in human male infertility. Three types of testicular samples representing normal spermatogenesis (NR), hypospermatogenesis (Hypo) and maturation arrest (MA) were subjected to analysis by real-time RT-PCR. The levels of *SEPTIN12* and *SEPTIN12- isoform 1* transcripts were significantly decreased in the testicular samples with spermatogenic defects (Figure 3A and B). We also analyzed semen samples from fertile men and men with asthenozoospermia using IFA and the anti-SEPT12 antibody. In humans, SEPT12 is located at the sperm neck, mitochondria, and annulus (Figure 3C). More than 100 sperm were evaluated for each person and a total of 3000 sperm were analyzed for their structural defects under the fluorescence microscope. The percentage of sperm with decreased SEPT12 expression was significantly increased in the men with asthenospermia (Figure 3D). In both groups (men with normal semen parameters and men

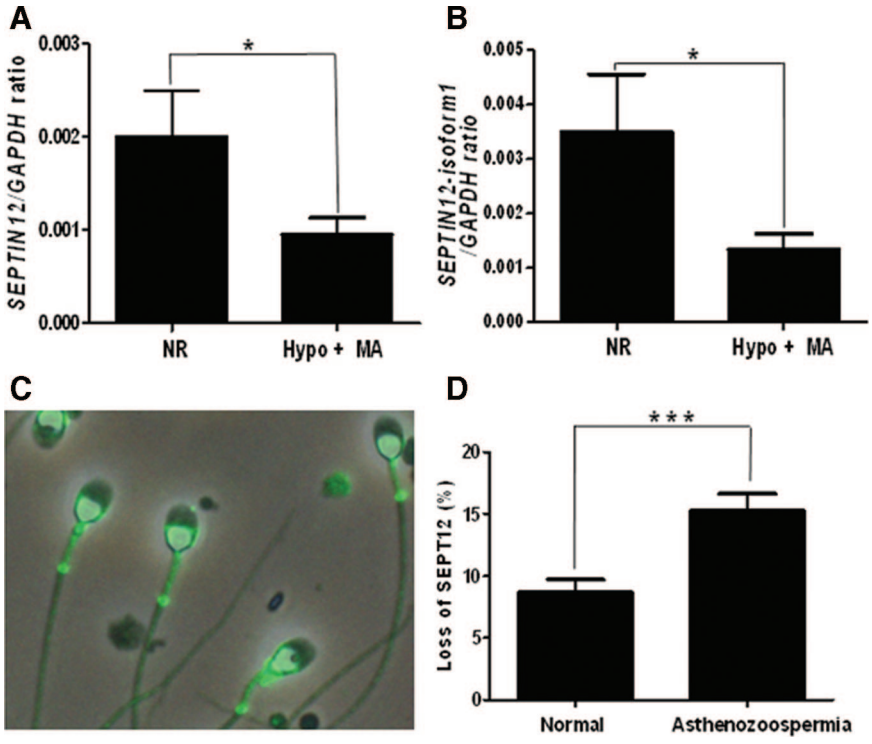

Figure 3. The expression of *SEPTIN12* decreases in infertile men. (**A** and **B**) The mRNA transcript amounts of *SEPTIN12* (shown as *SEPTIN12/GAPDH*) and *SEPTIN12-isoform1* (shown as *SEPTIN12-isoform1/GAPDH*) are significantly reduced in the testicular tissue of men with hypospermatogenesis (Hypo) and maturation arrest (MA) (*$P < 0.05$, *t*-test). **C:** In humans, SEPT12 proteins are mainly localized at sperm annulus and to a lesser extent at the midpiece and head. **D:** The percentage of sperm with loss of SEPT12 is increased in semen samples with asthenospermia ($n = 11$), as compared with fertile men with normal semen parameters (normal, $n = 19$). (**$P < 0.0001$, *t*-test).

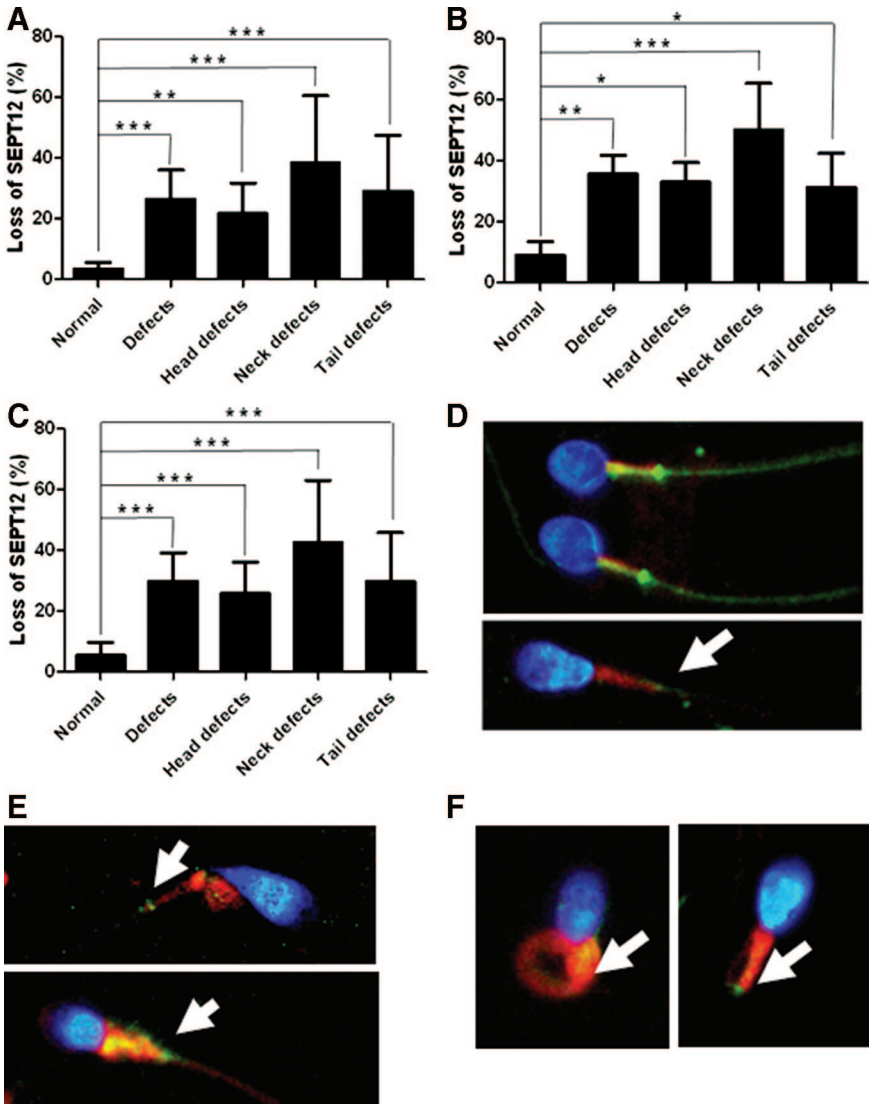

Figure 4. Decreased expression level of SEPT12 in spermatozoa with abnormal morphology. Loss of SEPT12 in spermatozoa with abnormal morphology (defects), including head defects (sperm with the head with abnormal shape or size), neck defects (sperm with bent neck or cytoplasmic droplet around the neck area), and tail defects (sperm with bent or coiled tail). The SEPT12 signals tend to be lost or dislocalized in abnormal sperm that were obtained from fertile men (**A**), infertile men with asthenospermia (**B**), and combination of both groups (**C**). $^{*}P < 0.05$; $^{**}P < 0.001$; $^{***}P < 0.0001$, one-way analysis of variance, Error bar indicate ±SEM. (**D**) shows SEPT12 signal in the normal sperm (**upper panel**). The SEPT12 signals tend to be lost or dislocalized in sperm with pyriform-like head (**D**, **arrow** at the **bottom panel**), sperm with bent neck (**E**, **arrow** at the **upper panel**), immature sperm with cytoplasmic droplet (**E**, **arrow** at the **bottom panel**), sperm with coiled tail (Figure F, **arrow** at the **left panel**), or sperm with bent tail (**F**, **arrow** at the **right panel**). SEPT12: green; Mitochondria: red; Nuclear DNA: blue.

with asthenospermia), sperm with head, neck or tail abnormalities tended to lose their SEPT12 signal (Figure 4, A–B). Because the two groups showed the similar trend, we pooled data from both groups (Figure 4C). Figure 4, D–F showed altered SEPT12 signals in sperm with various types of morphological defects.

Reproductive Phenotypes of Septin12$^{+/-}$ *Chimeric Mice*

To address the function of *Septin12* during spermiogenesis, we tried to knock out the *Septin12* locus in the mouse. First, we generated the *Septin12*$^{+/-}$ MESCs. The targeting vector was designed to replace exons 3 to 7 with a *neo* cassette (Figure 5A). Exons 3 to 7 were deleted after transfection of MESCs with a cre-recombinase vector. Successful targeting was validated by Southern blotting (Figure 5, B and a). The *Septin12*$^{+/-}$ MESCs were injected into C57BL/6 blastocysts, which were transferred to the uterus of pseudo-pregnant female mice. A total of 28 male and 9 female chimeric mice were generated. The genomic DNA of male chimeric mice was evaluated by Southern blot analysis after digestion with EcoRV (Figure 5, B and b). All of the chimeric mice were viable and appeared normal. The male chimeric mice (>6 weeks of age) were mated with C57BL/6 female mice for at least 3 months. Of 28 male chimeras, seven males fathered a total of 127 black progeny, and the other chimeric mice ($n = 21$) were infertile. Typing with microsatellite makers, the testicular tissues of chimeras contained the genetic background of both the C57BL/6 and the 129/Sv stains (Figure 5C).[27] Of 28 male chimeras, 16 were subjected to detailed analysis for their reproductive phenotypes and they were divided into two groups by the ratio of alleles: high for those with above 50% of the 129/Sv genomic DNA (±>50%, $n = 11$) and low for those with lower than 50% of the 129/Sv genomic DNA (±<50%, $n = 5$). The testis weights, sperm counts, and sperm motility of high- and low-chimera groups were significantly lowed than the wild-type controls (Figure 6A–D). Some semeniferous epitheliums were collapsed in both groups of chimeric mice. Some showed general-

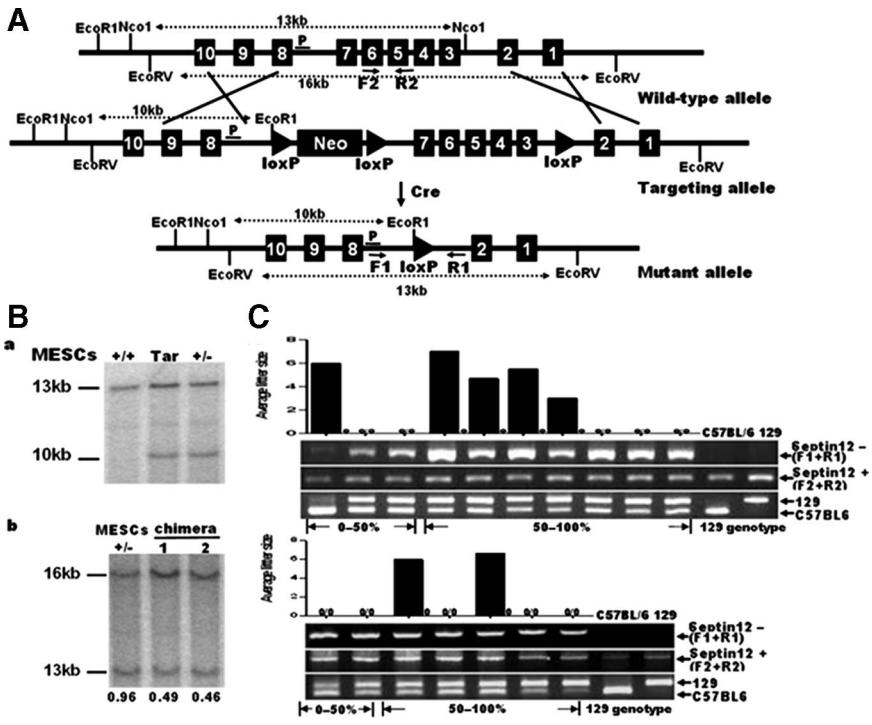

Figure 5. Targeted disruption of *Septin12* causes male infertility. **A:** Schematic representation of the strategy used to generate the *Septin12* mutant allele. Neo, neomycin; P: probe for Southern blotting; F1 and R1, primers for amplifying the mutant allele; F2 and R2, primers for amplifying the wild-type allele. **B:** Southern blot confirmation of the mutant allele in mouse embryonic stem cells (MESCs) and chimeric mice. **a:** MESC genomic DNA was digested with NcoI and EcoRI and blotted with probe P as indicated in (**A**). The 13kb and the 10kb fragments are from the wild-type allele and mutant allele, respectively. **b:** Southern blotting yields two bands (16 kb for the wild-type allele and 13 kb for the *Septin12* null allele) in both MESCs and the chimeric mice after enzymatic digestion by EcoRV. Ratios of the mutant and the wild-type allele are presented at the bottom. **C:** Fertility and genetic backgrounds of the chimeras. **Top**: Average litter size after mating with females for 3 to 12 months, 41% (7/28) of the chimeras sired black progeny (black bar) but 0% (0/28) chimeras sired agouti progeny. **Bottom**: Genotyping of the testicular DNA with the three sets of PCR primers, mutant allele (F1 and R1; *Septin12*$^{-}$) and wild-type allele (F2 and R2; *Septin12*$^{+}$) of *Septin12* and microsatellite markers to distinguish the genetic backgrounds of C57BL/6 and 129 strains.

ized loss of germ cells at all stages, reminiscent of the hypospermatogenesis-like phenotype in humans (Figure 6, E and F) while some showed developmental arrest of germ cells at the round spermatid stage, reminiscent of the maturation-arrest phenotype in humans (Figure 6G). Apoptosis signals were increased in the testicular sections of both the low- and the high-chimera groups (Figure 6, H–J).

Sperm Acrosome and Tail Defects in Septin12$^{+/-}$ *Chimera Mice*

To address the roles of *Septin12* during spermiogenesis, spermatozoa were isolated from cauda epididymis of high-chimera ($n = 11$), low-chimera ($n = 5$), and wild-type mice ($n = 14$). In the high-chimera groups, the sperm count was extremely low and almost all germ cells were immature. These immature germ cells might be exfoliated from the seminiferous tubules considering arrest of spermatogenesis at the round spermatid stage (the percentages of immature germ cells: wild-type: 0%, high-chimera 68.2 ± 45.9%; $P < 0.0001$). No mature spermatozoa were found in the vas deferens (Figure 7F and I). From immunostaining in Figure 2C, SEPT12 seemed to be involved in the acrosome and sperm tail formation. To investigate the acrosome integrity, an acrosome marker (lectin peanut agglutinin) was used to stain the testicular sections and spermatozoa. In the low-chimera groups, the acrosomes were loose and broken in germ cells from the testicular sections (Figure 7, A and B) or spermatozoa isolated from cauda epididymis (Figure 7, D and E). In the high-chimera groups, the acrosomes almost failed to form in germ cells from testicular sections (Figure 7C) or isolated from the cauda epididymis (Figure 7F) (the percentages of germ cells with abnormal acrosome morphology: wild-type: 20.4 ± 7.0%, low-chimera: 82.1 ± 24.7%, high-chimera: 83.2 ± 22.0.8%, $P < 0.05$ and $P < 0.0001$, respectively). In the low-chimera groups, the tails of most spermatozoa were bent or absent (arrows in Figure 7, G and H). The percentage of spermatozoa with tail defects was significantly increased in the low-chimera groups (wild-type: 10.9 ± 3.6%, low-chimera: 36.4 ± 6.0; $P < 0.05$). The sperm morphological defects of the chimeric mice are summarized in Table 1.

Ultrastructural Abnormalities of Spermatozoa in the Septin12$^{+/-}$ *Chimeric Mice*

Electron microscopy examination of spermatozoa purified from the cauda epididymis revealed additional ultrastructural abnormalities in the spermatozoa of the chimeras, including misshapen nuclei and disorganized mitochondria (Figure 8). The acrosome was broken and the mitochondria were loosely organized in the low-chimera groups (Figure 8, C–E), which are in sharp contrast to the wild-type sperm with integral acrosome and mitochondria architecture (Figure 8, A–B). Some spermatozoa exhibited disorganized mitochondria around the acrosome (Figure 8F). As shown in Figure 8, C and F, some vesicles were found in the sperm nuclei, suggesting nuclear damage. In the high-chimera groups, almost all germ cells found in the cauda epididymis were round spermatids without tail formation (Figure 8G). These results suggest SEPT12 may be essential for the formation of acrosome, mitochondrial, tail, and possibly nucleus during terminal differentiation of postmeiotic germ cells.

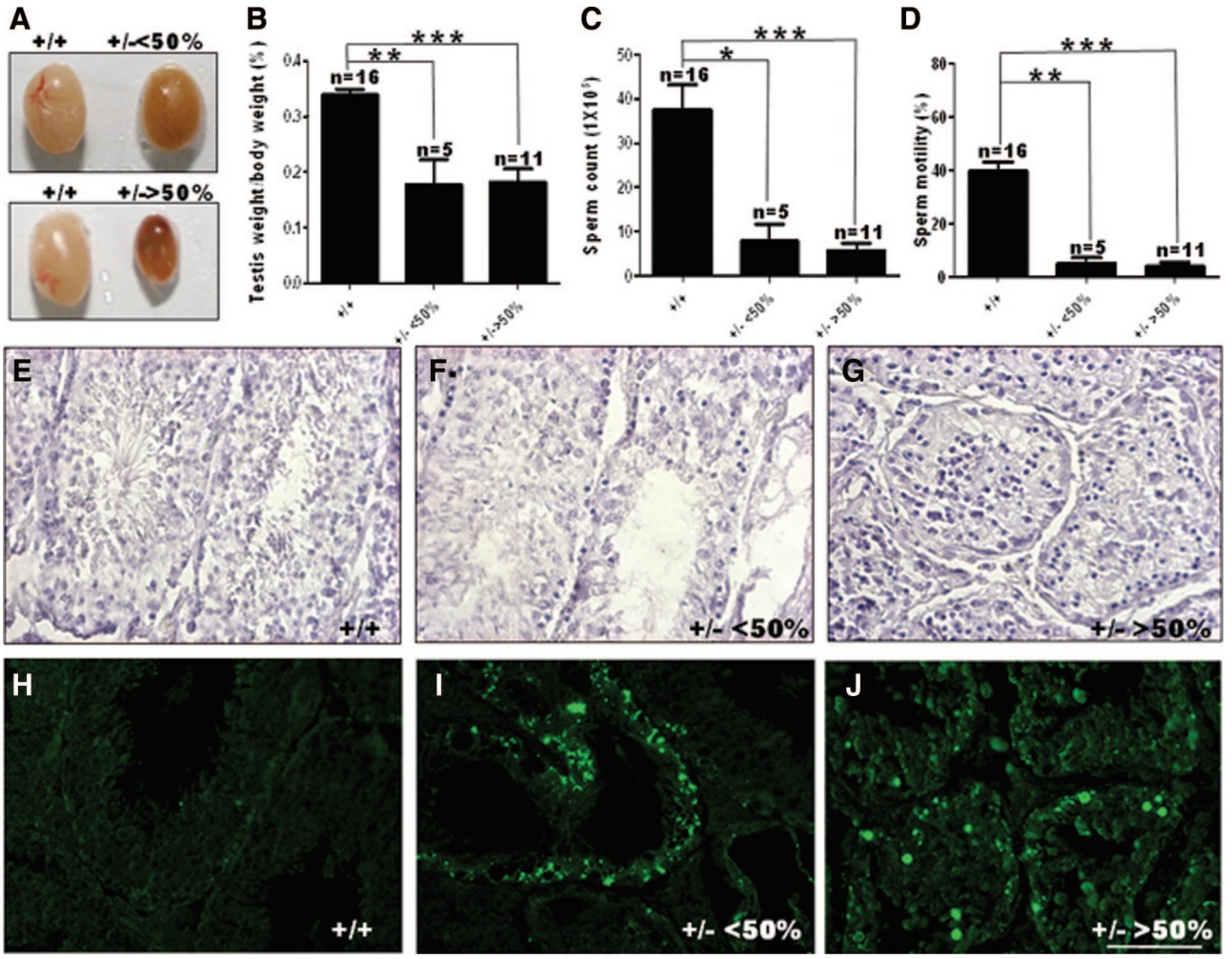

Figure 6. The reproductive phenotypes of chimeras. Chimeras were divided into two groups based on the ratio of 129/S versus C57BL6 genetic components in the testicular tissues: those with 129/Sv ±>50% and ±<50%. **A:** Representative testicular samples from the wild-type (WT) and chimeric mice, ±<50% (**upper, right**), and ±>50% (**bottom**, **right**). **B:** Chimeras had significantly smaller testes, shown as an index of testis weight versus body weight. **C** and **D:** The number of spermatozoa and percentages of mobile spermatozoa isolated from the vas deferens of chimeric mice were significantly lower than those of the wild-type mice. $^{*}P < 0.05$; $^{**}P < 0.001$; $^{***}P < 0.0001$; Error bars indicate ±SEM. **E–G:** Histological analyses of testes from wild-type (**E**), ±<50% chimera (**F**), and ±>50% chimera (**G**). **F:** The numbers of male germ cell were significantly deceased in ±<50% chimeras compared with the wild-type mice (**E**). The spermatogenesis was arrested at the spermatid stage in the ±>50% chimeric mice (**F**). **H–J:** Apoptosis signals (green) were increased in testicular sections from ±<50% chimeric (**I**) and ±>50% chimeric (**J**) mice than the wild-type mice (**H**). Scale bar = 50 μm.

Discussion

Septins are required for the completion of cytokinesis in somatic cells. Loss of septin function results in multinuclear phenotypes from yeast to mammals, suggesting highly conservative role of septins during evolution. In yeast, the plasma membrane is compartmentalized into the bud-mother rings that are separated by a septin-dependent diffusion barrier.[29–31] The main phenotype of the original temperature-sensitive mutants (*cdc3, cdc10*, *cdc11,* and *cdc12*) is a lack of bud-neck filaments and multinuclear defects.[3,32,33] Growing evidence has suggested mammalian septins interact with diverse molecules to ensure completion of cytokinesis in somatic cells.[34,35] In somatic cells, the mother-daughter cells are connected transiently by an intercellular bridge at the termination of cytokinesis, followed by abscission, or cutting of midbody channel. In germ cells, the intercellular bridges of germ cells are transformed into a stable structure. The germ cell intercellular bridge is evolutionarily conserved from invertebrate to mammals. Disruption of structural components of the intercellular bridge will result in sterility.[36] In *Drosophila*, septins are involved in the formation of the structure between the incomplete cytokinesis of male and female germ cells, ring canals structure.[5] In mammalian species, septins are components of intercellular bridges of germ cells. Of 13 septins, three (SEPT 2, 7, and 9) have been found to co-localize with an intercellular bridge marker of male germ cells, TEX14 (*testis-expressed gene14*), in newly forming intercellular bridge.[36,37] However, SEPT12 does not seem to be a component of the intercellular bridges based on our results.

Mature spermatozoa consist of four major compartments: acrosome, nucleus, midpiece, and tail. How these

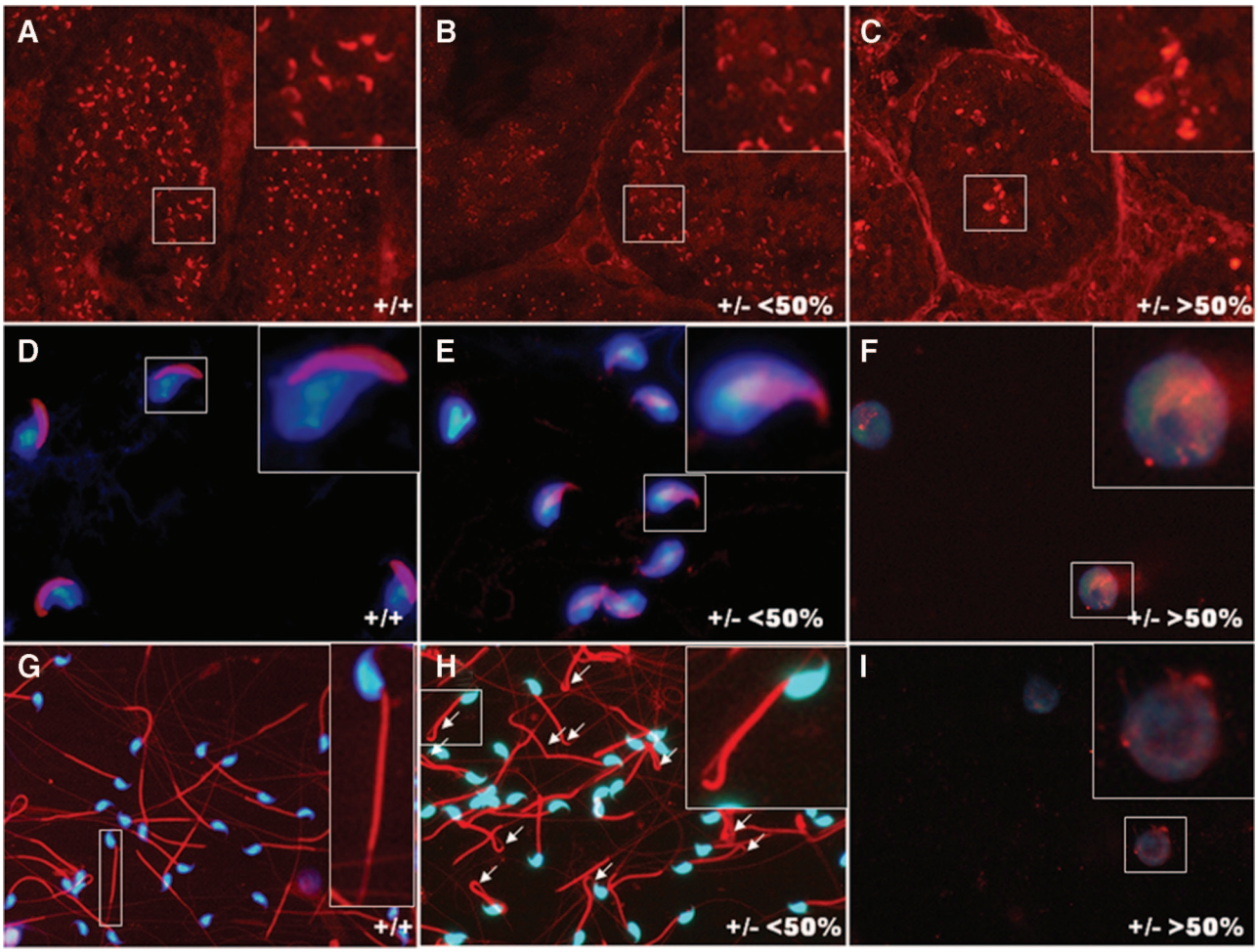

Figure 7. The reproductive phenotypes of chimeras. Immunofluorescent staining of the testicular sections using an acrosome marker for the wild-type (WT) (**A**), ±<50% chimeric (**B**), and ±>50% chimeric (**C**) mice. Both groups of chimeras showed significant fractions of germ cells with abnormal acrosome signals. The spermatozoa were analyzed using the same acrosome marker: spermatozoa isolated from vas deferens of wild-type (**D** and **G**), ±<50% chimeric (**E** and **H**), and ±>50% chimeric (**F** and **I**) mice. Enlarged figures shown in **right upper** or **lower** corner (**A–I**). The **arrow** indicates spermatozoa with bent tail (**H**), as compared with normal spermatozoa (**G**). Acrsome marker: red (**A–F, I**); Mitochondria marker: red (**G** and **H**); Nuclear DNA: blue (**A–I**). Magnification = original ×400 in **A–I.**

compartments form during terminal differentiation still remains obscure. In this study, we found SEPT12 protein widely expressed and forms ring-like structure in different locations of postmeiotic germ cells during the terminal differentiation processes, including the peri-acrosome area, peri-nuclear area, midpiece, and tail (Figure 2C). Although our chimeric mice shared some phenotypes reminiscent of those for the *Septin4* KO mice, obvious differences do exist between the mouse models of these two genes. For the *Septin4*, the reproductive phenotypes, including bent-tail, as well as acrosome and mitochondrial dysfunction in mature spermatozoa, were observed only in the *Septin4*$^{-/-}$ mice.[10,11] However, the reproductive effect is far more profound for SEPT4 deficiency. We found haploinsufficiency of *Septin12* is sufficient to cause severe defects of both mature and immature germ cells. Septins usually mediate their cellular functions through the formation of macromolecular and hetero-oligomeric filaments with other family members.[38] It is highly likely that SEPT12 cooperates in interactions with fixed stoichiom-

Table 1. The Percentages of Abnormal Spermatozoa from the Cauda Epidydimis of Mice

Phenotype	Wild-type (n = 14)	Chimera ± <50% (n = 5)	Chimera ± >50% (n = 11)
Round spermatid	0%	30.4 ± 35.1%	68.2 ± 45.9%‡
Abnormal acrosome morphology§	20.4 ± 7.0%	82.1 ± 24.7%*	83.2 ± 22.0%‡
Headless	8.6 ± 4.1%	25.5 ± 16.3%	19.0 ± 7.6%*
Bent neck	4.5 ± 4.1%	3.3 ± 3.2%	7.4.0 ± 4.4%
Tail defects¶	10.9 ± 3.6%	36.4 ± 6.0%*	38.4 ± 9.7%†

*$P < 0.05$; †$P < 0.001$; ‡$P < 0.0001$.
§Diagnosis of abnormal acrosome morphology was made by staining with an acrosome marker (lectin PNA).
¶Tail defects include bent and lost tail of sperm.

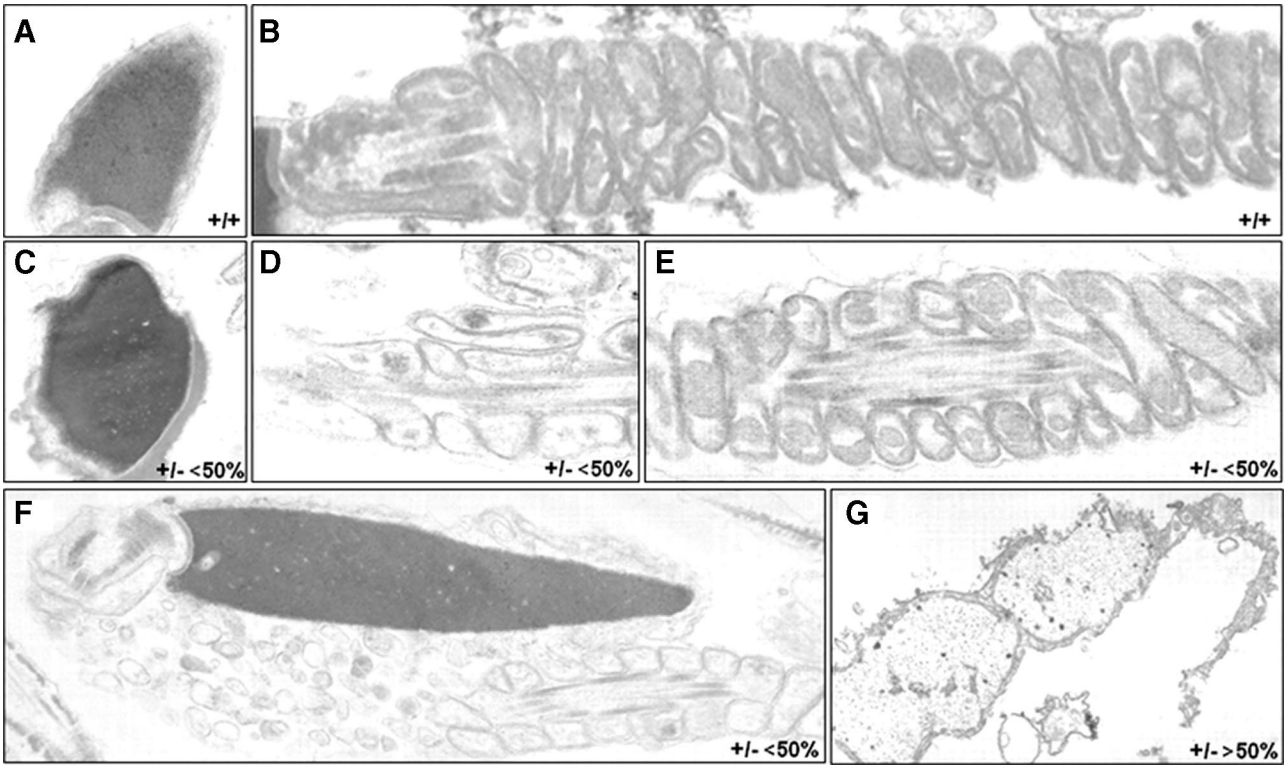

Figure 8. Electron microscopy shows defects in sperm acrosome and mitochondrial architecture in the chimeric mice. The spermatozoa purified from the epididymal cauda of wild-type (WT) (**A** and **B**), ±<50% chimera (**C–F**), and ±>50% chimera (**G**). The spermatozoa of ±<50% chimeric mice exhibit loss or morphological defects of the acrosome (**C**), as compared with the wild-type mice (**A**). The sperm from ±<50% chimera had severely reduced membranous material of mitochondria (**D–E**) relative to the wild-type (**B**). Spermatozoa from the low-chimera group (±<50%) exhibit a disorganized midpiece attached around an abnormally shaped head with defective acrosome (**F**). **G:** Immature spermatids without tail and acrosome formation. Magnification = original ×12,000 in **A** and **B**; ×15,000 in **C, D, E** and **F;** ×20,000 in **F**; and ×10,000 in **G**.

etry with other SEPTINS (eg, SEPT4).[27,30] Considering the overt dosage-sensitive effect, SEPT12 may play a pivotal role in the complex formation during spermiogenesis.

The causes of infertility could not be identified in the majority of men with spermatogenic defects.[39] The pathology of male infertility includes anatomical defects, gametogenesis dysfunction, endocrinopathies, immunological problems, ejaculatory failure, environmental exposures, and gene mutations.[40–43] In this study, we found men with two distinct histological patterns of spermatogenic defects (maturation arrest and hypospermatogenesis) had decreased *SEPTIN12* transcript levels in their testicular tissues. Because *SEPTIN12* is only expressed in postmeiotic germ cells, decreased transcript levels may be secondary to the loss of postmeiotic germ cells in the testicular specimens. Intriguingly, we found SEPT12 signal was decreased in sperm samples of asthenospermic men. In addition, sperm with abnormal head, neck and tail morphology tend to have reduced SEPT12 expression level. In humans, the percentage of sperm with abnormal morphology ranges from 2% to 18.7% in fertile men. The percentage increased from 6% to 20% in the infertile men. Our finding suggests defective synthesis, increased degradation, or dysfunction of SEPT12 may be causally related to both motility and morphological defects of sperm. Our finding is also in accordance with the hypothesis that SEPT12 is involved in compartment formation of male germ cells.

Knockout mouse models have significantly enhanced our knowledge regarding the regulatory pathways involved in mammalian spermatogenesis. To date, more than 300 knockout mouse models have been found to be associated with testicular phenotypes, including loss of germ-line stem cells, maturation arrest at spermatocyte or spermatids, impaired sperm motility and abnormal sperm flagellum structure.[39,40,43] In knockout mice, haploinsufficiency of the following genes have been reported to cause testicular failure: *Kelch homolog 10*, *Cytochrome P450/17-hydroxylase/17,20-lyase*, *Testis specific ser/thr kinase 1& 2, Paralyzed flagella 20*, *Protamine1*, *Protamine2*, and *Heterogeneous ribonucleoproteins G expressed in the Testis*.[27,44–48] PF20 is a critical structure component of the axoneme central apparatus. The phenotype of chimeric mice carrying one mutant *Paralyzed flagella 20* allele had impaired spermatogenesis with a significant loss of germ cells at the round spermatid stage and disorganization of sperm axoneme structure.[48] In this study, we found haploinsufficiency of *Septin12* disrupts spermiogenesis. Septins are generally regarded as cytoskeletal proteins considering the effect on various cellular functions through interaction with tubulin or actin.[6,49] SEPT12 is therefore a second cytoskeletal protein (next to Paralyzed flagella 20) haploinsufficiency of which would disrupt spermatogenesis. Of all family members, four septin genes have been targeted in the mouse. *Septin5*, *Septin3* and *Septin6* deficiencies do not show any overt pheno-

types, implying a high degree of functional redundancy in the SEPTIN system except *Septin4*.[10,11,50–52] SEPT12 thus becomes an ideal target for male contraception considering two of its prominent features-tissue-specificity and dosage-sensitivity.

Taken together, this study shows an important role of *Septin12* expression level in mammalian spermatogenesis. Our study suggests a novel role of the septin gene family in forming subcellular compartments of germ cells. Our data also indicates haploinsufficiency of *Septin12* disrupts spermiogenesis in mice. The findings in human testicular and semen samples are consistent with those from the mouse study, suggesting conserved roles of SEPT12 in mammalian species. Given the distinct reproductive phenotype of the *Septin12*$^{+/-}$ chimeric mice, *SEPTIN12* seems to be a good candidate for sterile gene in humans.

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