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# *Catsper3* and *Catsper4* Are Essential for Sperm Hyperactivated Motility and Male Fertility in the Mouse[1]

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## ABSTRACT

***Catsper3* and *Catsper4* are two recently identified testis-specific genes homologous to *Catsper1* and *Catsper2* that have been shown to play an essential role in sperm hyperactivated motility and male fertility in mice. Here we report that *Catsper3* and *Catsper4* knockout male mice are completely infertile due to a quick loss of motility and a lack of hyperactivated motility under capacitating conditions. Our data demonstrate that both CATSPER3 and CATSPER4 are required for hyperactivated sperm motility during capacitation and for male fertility. The present study also demands a revisit to the idiopathic male infertility patients who show normal sperm counts and normal initial motility for defects in sperm hyperactivated motility and for potential CATSPER gene mutations. The CATSPER channel also may be an excellent drug target for male contraceptives.**

*fertilization, infertility, ion channel, sperm, spermatogenesis, sperm capacitation, sperm maturation, sperm motility and transport, testis*

## INTRODUCTION

Motility is essential for sperm to reach and fertilize eggs in mammals. Spermatozoa start to gain motility during their maturation in the epididymis [1–3]. When spermatozoa are in the cauda epididymis and the vas deferens, they display a sinusoidal movement pattern characterized by a symmetrical tail motion with high frequency and low amplitude [4]. Soon after spermatozoa are exposed to the environment of the female reproductive tract, they change to a pattern of asymmetrical motion with lower frequency and higher amplitude, which is termed *hyperactivated motility* [4–8]. The hyperactivated motility may help spermatozoa swim faster and generate enough force to penetrate cumulus cells and zone pellucida during fertilization. The acquisition of hyperactivated motility correlates with an elevation of intracellular $Ca^{2+}$ concentration [9, 10], suggesting that ion channels are involved in this process. Although numerous ion channels have been detected in various regions of sperm [11–14], studies using gene knockout mice over the past decade demonstrate that CATSPER1 and CATSPER2 are the only two putative cation channels essential for the hyperactivated motility [15–19]. Male mice deficient in either the *Catsper1* or the *Catsper2* gene are completely infertile, and neither *Catsper1*-null nor *Catsper2*-null sperm display hyperactivated motility in capacitating conditions. A high degree of structural homology between CATSPER1 and CATSPER2 and an identical phenotype in *Catsper1* and *Catsper2* mice [20] strongly suggest that they may be components of the same channel responsible for the hyperactivated motility of spermatozoa.

The recent completion of the mouse and human genome projects facilitated the identification of the majority, if not all, of the protein-encoding genes. High throughput expression profiling analyses, including microarray and in silico database mining, have aided in the discovery of numerous genes that are exclusively expressed during male germ cell development [21, 22]. We previously identified *Catsper3* and *Catsper4* using an in silico database mining strategy, and we showed that these two proteins display many similarities with CATSPER1 and CATSPER2 in domain structures and expression profiles [23]. The onset of mRNA expression of all four *Catsper* genes is in round spermatids, and proteins are synthesized in elongated spermatids [15, 23, 24]. Highly homologous domain structures and almost identical expression patterns suggest that the functions of the four CATSPER proteins may be overlapping. To define the physiologic roles of the newly identified two *Catsper* genes, we generated mouse lines lacking either *Catsper3* or *Catsper4*. We here report that *Catsper3* and *Catsper4* knockout male mice display an identical phenotype characterized by infertility due to a lack of hyperactivated sperm motility.

## MATERIALS AND METHODS

### *Generation and Genotype Analyses of* Catsper3 *and* Catsper4 *Knockout Mice*

*Catsper3* and *Catsper4* genomic fragments were isolated from a mouse genomic library (from the 129S6/SvEv strain) and were analyzed by restriction enzyme digestion and sequencing. A targeting vector for generating a null *Catsper3* allele was constructed such that exons 4 and 5 were replaced with a *Pgk/HPRT* minigene cassette after homologous recombination (Fig. 1A). Linearized targeting vectors were electroporated into the AB2.2 embryonic stem (ES) cells, and HAT-FIAU double-selected ES cell clones were screened by Southern blot analysis as described previously [25, 26]. Two ES cell clones carrying the mutant *Catsper3* allele were injected into C57BL/6J blastocysts and implanted into pseudopregnant females ($F_1$ females of CBA crossed with 129 Sv/Ev). The resulting chimeras were mated to C57BL/6J mice to produce $F_1$ progeny with heterozygous genotype (*Catsper3*$^{+/-}$). Further crosses between the *Catsper3*$^{+/-}$ mice yielded $F_2$ homozygous mutants (*Catsper3*$^{-/-}$). Genotyping of $F_1$ mice was performed on DNA from tail tips by Southern blot analysis (see below). After $F_2$, PCR-based genotyping was performed using primers specific to the WT allele (upstream: 5′-CTGTCCGTGACACAAAATGG-3′; downstream: 5′-AACCCACTCCAGAACATTCG-3′) and primers specific for the KO allele (upstream: 5′-TAAAGCGCATGCTCCAGACT-3′; downstream: 5′-TCTCCTCCATAATCGCAAGG-3′).

Similarly, a targeting vector for generating a *Catsper4*-null allele was constructed such that exons 1–3 would be deleted after homologous recombination. Electroporation, screening of correctly targeted ES cell clones, microinjection, and breeding were performed as described above. Genotyping of $F_1$ mice was performed using Southern blot analysis (see below). For genotyping mice after $F_2$, PCR was employed using primers specific for the wild-type (WT) alleles (upstream: 5′-CTTGGTCTCCCCTTCACTCA-3′;

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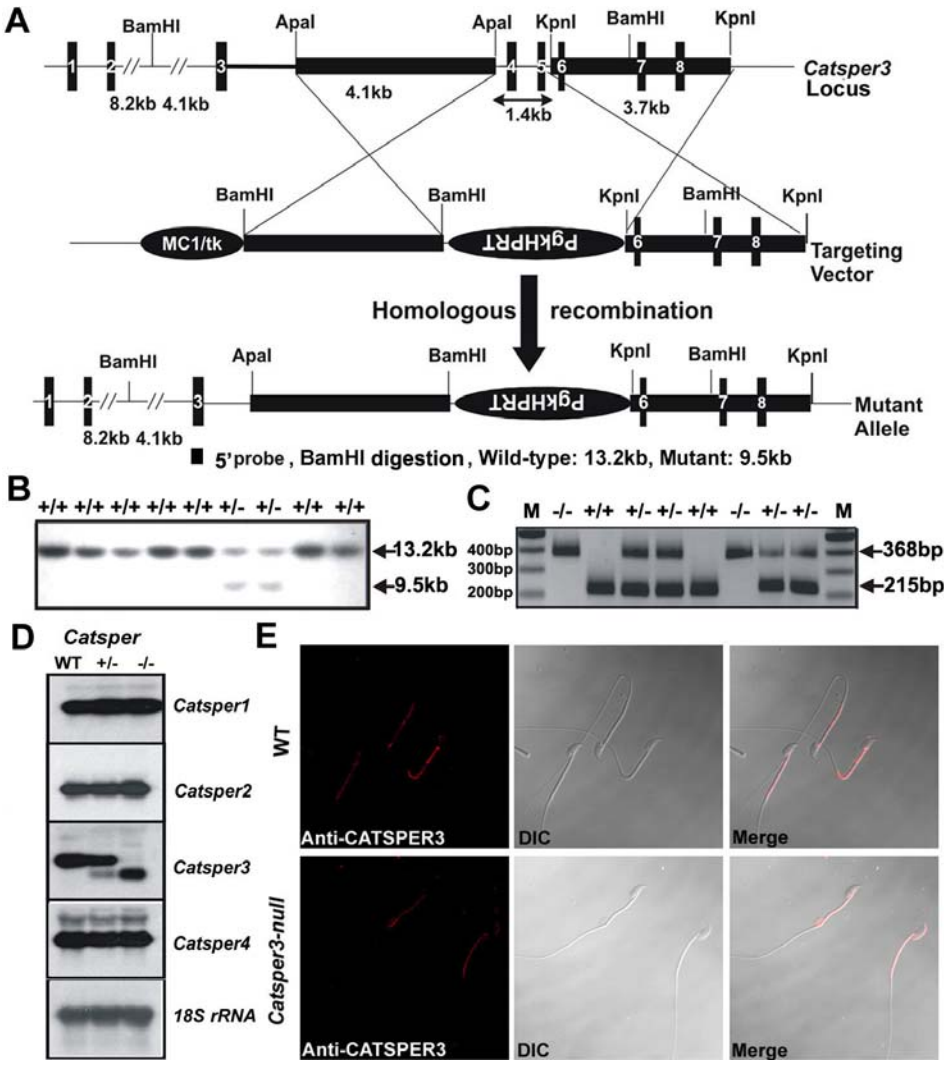


FIG. 1. Generation of *Catsper3* knockout mice. **A**) Strategy of generating a functionally null *Catsper3* allele via homologous recombination. **B**) A representative Southern blot result showing two ES clones carrying the mutant allele. A 5′ external probe detected the WT allele as a 13.2-kb band and the mutant allele as a 9.5-kb band. **C**) PCR-based genotyping analysis on a litter of eight pups from heterozygous breeding pair (*Catsper3*$^{+/-}$ × *Catsper3*$^{+/-}$). The primer set used detected the WT allele as a 215-bp PCR product and the mutant allele as a 369-bp PCR product. **D**) Northern blot analysis of the expression levels of *Catsper1*, *Catsper2*, *Catsper3*, and *Catsper4* in WT, *Catsper3*$^{+/-}$, and *Catsper3*$^{-/-}$ mouse testes. Levels of *18S rRNA* were used as a loading control. **E**) Immunofluorescent staining of CATSPER3 in spermatozoa collected from WT and *Catsper3*-null mice. Red color representing specific immunoreactivity was detected mainly in the principal piece of the sperm tail and occasionally on the acrosome of sperm. Since only the pore domain of CATSPER3 was deleted, the polyclonal anti-CATSPER3 antibody could still recognize other epitopes of the protein. Original magnification ×400.

downstream: 5′-TCCTTCTCTCTCCCCCTTGT-3′) and primers for the KO allele (upstream: 5′-CTTGGTCTCCCCTTCACTCA-3′; downstream: 5′-TAAAGCGCATGCTCCAGACT-3′).

All procedures used in generating and analyzing mutant mice were approved by the Institutional Animal Care and Use Committee of the University of Nevada, Reno. The mice used in this study were the offspring of $F_1$ and/or $F_2$ animals on a 129S6Sv/Ev- C57BL/6J mixed background.

## Southern Blot Analysis

DNA prepared from ES cells and tail tips was digested with restriction enzymes (*Bam*HI for *Catsper3* and *Hin*dIII for *Catsper4*), fractionated by agarose gel electrophoresis, transferred to a Hybond-N membrane (Amersham Biosciences Inc., Little Chalfont, UK), and then hybridized with DNA probes labeled with [α-P$^{32}$]-dCTP using the Amersham Rediprime II Ramdom Prime labeling system (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). The 181-bp-long 5′ external probe for *Catsper3* was amplified from genomic DNA using primers (upstream: 5′-CCTCATGAAGGTCTATGTGGA-3′; downstream: 5′-GGAGATAAGCTTGAGGATTCG-3′) flanking the 5′ homology region (Fig. 1A). The 112-bp 3′ external probe for *Catsper4* was generated using primers (upstream: 5′- GTAGAAACATTACGAGCTATTCTCGACC-3′; downstream: 5′- GCTCACCTTCCAGAAAATCCAGAAG-3′) flanking the 3′ homology region (Fig. 2A). Hybridization was performed overnight at 58°C in Perfect Hyb Plus Hybridization Buffer (Sigma-Aldrich Co., St. Louis, MO). Filters were washed at 58°C in 2× SSC (0.15 M sodium chloride/0.015 M sodium citrate, pH 7.0) with 0.1% SDS for 30 min, followed by two washes in 0.2× SSC containing 0.1% SDS for 30 min each. Expected band sizes for the WT and mutant alleles of *Catsper3* are 13.2 kb and 9.5 kb, respectively. Expected sizes for the WT and mutant alleles of *Catsper4* are 14.9 kb and 10.8 kb, respectively.

## Northern Blot Analyses

Northern blot analyses were performed as described previously [23]. Complementary DNA probes were generated by RT-PCR (primer sequences available upon request) using testicular total RNA, and positions of the probes in the full-length cDNAs are as follows: the *Catsper*1 probe corresponds to nucleotides 775-1925 bp of NM_139301, the *Catsper2* probe to nucleotides195-1757 of NM_153075, the *Catsper*3 to nucleotides 98–1148 of NM_029772, and the *Catsper4* to nucleotides 151-1319 of NM_177866.

## Histology Examination

Testes and epididymides were dissected, fixed in the Bouin fixative (Sigma-Aldrich Co.), and embedded in paraffin. Sections (5 μm) were prepared and stained with hematoxylin and eosin.

## Immunofluorescent Staining of Sperm Smears

Cauda epididymal sperm were spread onto Superfrost Plus slides (Fisher Scientific, Hampton, NH) and air dried. Slides of sperm smears were stained using an affinity-purified polyclonal anti-CATSPER3 antibody (1:200 dilution) and an affinity-purified rabbit polyclonal anti-CATSPER4 antibody (1:200 dilution) prepared using a synthetic peptide corresponding to amino acid residues 243–260 (YTDFQMDEREYAMEVGGA; Bio-Synthesis Inc., Lewisville, TX) using procedures described previously [23]. Images of fluorescence and differential contrast were acquired using a confocal laser scanning microscope and its imaging system (LSM500; Carl Zeiss Microimaging, Thornwood, NY).

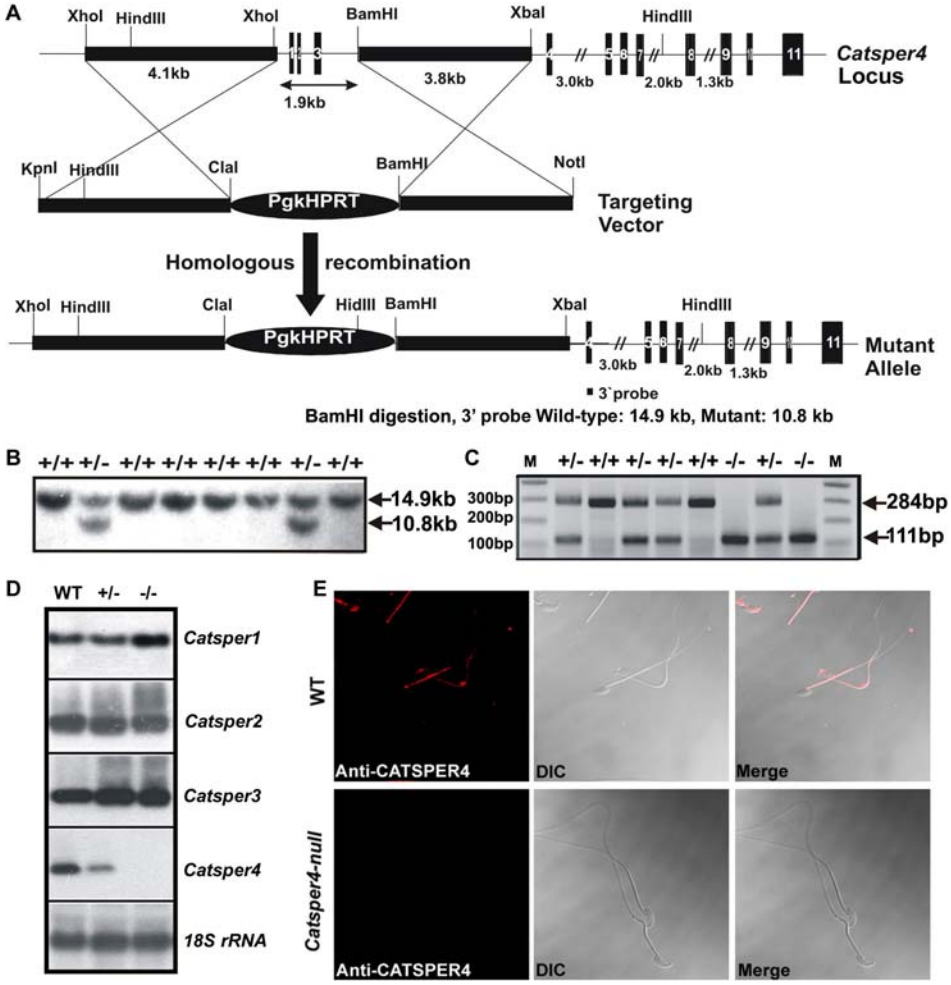


FIG. 2. Generation of *Catsper4* knockout mice. **A**) Targeting strategy to completely inactivate the *Catsper4* allele via homologous recombination. Since exon 1 contains the transcription start codon, deletion of exons 1–3 would completely abolish the transcription, and thus generate a *Catsper4*-null allele after homologous recombination. **B**) A representative Southern blot result showing two ES clones carrying the mutant allele. A 3′ external probe detected the WT allele as a 14.9-kb band and the mutant allele as a 10.8-kb band. **C**) PCR-based genotyping analysis on a litter of eight pups from heterozygous breeding pair (*Catsper4*$^{+/-}$ × *Catsper3*$^{+/-}$). The primer set used detected the WT allele as a 284-bp PCR product and the mutant allele as a 111-bp PCR product. **D**) Northern blot analyses on the expression levels of *Catsper1*, *Catsper2*, *Catsper3*, and *Catsper4* in WT, *Catsper4*$^{+/-}$, and *Catsper4*$^{-/-}$ mouse testes. Note a complete lack of mRNA expression of *Catsper4* in the *Catsper4*$^{-/-}$ mouse testes. Levels of *18S rRNA* were used as a loading control. **E**) Immunofluorescent staining of CATSPER4 in spermatozoa collected from WT and *Catsper4*-null mice. Red color representing specific immunoreactivity was detected mainly in the principal piece of WT sperm tails and occasionally on the acrosome of WT sperm. Sperm from the *Catsper4*-null mice showed no immunoreactivity. Original magnification ×400.

### *Sperm Preparation and Incubation*

Cauda epididymides and vas deferens were excised from male mice. After a rinse with HS solution (135 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 20 mM HEPES, 5 mM glucose, 10 mM lactic acid, and 1 mM sodium pyruvate, pH 7.40), sperm were collected into either HS medium or HTF medium (2.0 mM $CaCl_2$-$2H_2O$, 2.5 mM glucose, 5.0 mM KCl, 0.4 mM $KH_2PO_4$, 0.2 mM $MgSO_4$-$7H_2O$, 100 mM NaCl, 25 mM $NaCHO_3$, 18.5 mM sodium lactate, 0.3 mM sodium pyruvate, 0.2 mM penicillin G sodium salt, 0.3 mM streptomycine sulfate, 4.00 g/l BSA, and 2 mg/l phenol red), or divalent-free (DVF) medium (150 mM sodium gluconate, 20 mM HEPES and 5 mM $Na_3$HEDTA, and 5 mM glucose; pH 7.4). All chemicals used for the preparation of medium solutions were purchased from Sigma-Aldrich Co.

### *Analysis for Sperm Motility*

Motility assays were performed at room temperature. An aliquot of sperm suspension (~5 μl) was loaded onto a chamber slide with 20-μm depth (Conception Technologies, San Diego, CA) and subjected to microscopic observation under a dark or phase-contrast field. Video clips of sperm motility were acquired using a CCTV video camera (Bio-Rad Laboratories Inc., Hercules, CA) mounted on an upright microscope (AxioSkope 2 Plus; Carl Zeiss MicroImaging Inc.). The video stream was digitized using a Canopus analogue to digital converter box (Canopus Corp., San Jose, CA) and was recorded onto an iMac computer (720 × 480 at 30 frames per second) using iMovie software (Apple, Cupertino, CA). A novel CASA system based on open source software [27] was employed to analyze the video clips of ~60-sec duration each. Parameters that were examined included percentage of motile sperm cells, path velocity (the distance traveled along a smoothed average path divided by the elapsed time), linear velocity (the straight line distance between the first and final locations of the sperm head divided by the elapsed time), track velocity (the total distance traveled by the sperm head from image to image divided by the elapsed time), and straightness (linear velocity divided by path velocity multiplied by 100; higher values indicate a straighter trajectory). In the viscosity experiments, sperm were suspended in 7% polyvinylpyrrolidone (PVP) in HTF. An aliquot of 80 μl of spermatozoa was put on a slide, and a coverslip then was placed over the sample. Analyses were performed as described above. Three to five mice of each genotype were analyzed.

### *Whole-Cell Patch Clamp*

Corpus epididymal spermatozoa were prepared as described above in HS medium. Whole-cell currents were recorded at room temperature using the standard patch clamp technique with an Axopatch 200B amplifier (Axon Instruments, Union City, CA). Borosilicate pipettes with a resistance of 4–10 MΩ were filled with a solution containing the following (in mM): 135 caesium methanesulphonate, 5 CsCl, 10 HEPES, 10 EGTA, 5 $Na_2$ATP, and 0.5 $Na_2$GTP, pH 7.2). Bath solutions used included: (1) HS solution, (2) DVF solution, and (3) low-$Ca^{2+}$ solution (2 mM $CaCl_2$ was added into the DVF solutions to obtain ~3.3 μM free external $Ca^{2+}$ concentration [DVF + Ca solution] in accordance with the Sliders v2.00 program [Chris Patton, Stanford University, Stanford, CA]). Osmolarity of these solutions was about 305 mmol $kg^{-1}$. Signals were acquired using the PClamp 9.02 (Axon Instruments) and were filtered at 2 kHz and sampled at 4 kHz.

### *Statistic Analyses*

Data are shown as the mean ± SEM, and statistical differences between data sets were assessed by the Student *t*-test using Origin 6.0 (Microcal Software Inc., Northampton, MA), and significance was accepted at the level of $P < 0.05$.

## RESULTS

### *Generation of* Catsper3 *and* Catsper4 *Knockout Mice*

*Catsper3* consists of eight exons spanning ~24 kb on the mouse chromosome 13. We obtained a genomic fragment

containing exons 4–8 by screening a mouse genomic library (129 Sv/Ev strain). A targeting construct was designed to replace exons 4 and 5 with a *Pgk*/*HPRT* minigene cassette after homologous recombination in the AB2.2 mouse ES cells (Fig. 1A). The exons 4 and 5 of *Catsper3* encode amino acids 152–257 of CATSPER3, which contains the pore-forming region located between the fifth and sixth transmembrane domains of CATSPER3. Since ions are supposed to get into the cell through the pore, deletion of this region would result in a nonfunctional channel, and thus this mutation is functionally null. Southern blot analyses using a 5′ external probe identified a total of four ES cell clones with correct targeting (Fig. 1B), and two clones were injected into blastocysts (from C57 BL/6 strain) and implanted into the uterus of pseudopregnant recipient mice. $F_1$ heterozygous (*Catsper3*$^{+/-}$) males were obtained by breeding the WT females (C57 BL/6) with the chimeric mice, and intercrossing between $F_1$ heterozygous mice produced homozygous mice (*Catsper3*$^{-/-}$). Once the germline transmission was confirmed by Southern blot analyses, a PCR-based method was used for genotyping using tail DNAs (Fig. 1C). Northern blot analyses revealed that the *Catsper3* transcript was truncated (Fig. 1D), and sequencing of the PCR fragment encompassing the deleted region verified that the mRNA transcribed from the mutant *Catsper3* allele lacked exons 4 and 5 (data not shown). Immunofluorescent staining of *Catsper3*-null and WT sperm using a polyclonal anti-CATSPER3 antibody detected stronger immunoreactivity in the principal piece of the sperm tail, and weaker immunoreactivity was seen occasionally on the acrosome (Fig. 1E). Since the mutant *Catsper3* transcript lacked exons 4 and 5, the resultant mutant protein still contained epitopes that could be recognized by the polyclonal antibody used.

*Catsper4* is located on mouse chromosome 4 and consists of 11 exons spanning 15 kb. Using a genomic fragment containing the first three exons obtained by screening a mouse genomic library, we constructed a targeting vector that would replace exons 1–3 with the *Pgk*/*HPRT* minigene cassette after homologous recombination in AB2.2 ES cells (Fig. 2A). Southern blot analyses using a 3′ external probe identified a total of eight ES cell clones with correct targeting (Fig. 2B), and two clones were injected into blastocysts for production of chimeras. PCR was used for genotyping analyses after confirming germline transmission of the mutant allele using Southern blot analyses for $F_2$ mice (Fig. 2C). Since the transcription start codon is located in exon 1, deletion of exons 1–3 would completely abolish the transcription of *Catsper4,* and thus generate a null allele. As expected, Northern blot analyses on testicular total RNAs verified that the *Catsper4*$^{-/-}$ mice expressed no *Catsper4* transcript. Thus, we have generated mutant mice with a complete inactivation of the *Catsper4* gene. Using a polyclonal anti-CATSPER4 antibody, stronger immunoreactivity was detected in the principal piece of the WT sperm tail, and the acrosomes of some WT sperm also showed immunoreactivity (Fig. 2E, upper panels). The *Catsper4*-null sperm lacked staining, further demonstrating that *Catsper4* gene was completely inactivated in the *Catsper4*$^{-/-}$ mice (Fig. 2E, lower panels).

### Catsper3$^{-/-}$ *and* Catsper4$^{-/-}$ *Males Are Infertile*

Breeding experiments using heterozygous mating pairs demonstrated that the *Catsper*3 and the *Catsper4* mutant alleles were inherited in Mendelian fashion, suggesting that null mutations in either *Catsper3* or *Catsper4* did not affect embryonic development (data not shown). *Catsper3*$^{-/-}$ and *Catsper4*$^{-/-}$ females were fertile, and no differences were observed in litter size and litter intervals compared with WT females (data not shown). Both *Catsper3*$^{-/-}$ and *Catsper4*$^{-/-}$ mice were grossly indistinguishable from their WT littermates. These data are consistent with the confined expression of *Catsper3* and *Catsper4* to the testis. Adult *Catsper3*$^{-/-}$ and *Catsper4*$^{-/-}$ males bred with adult WT females for more than 4 mo did not sire any offspring, despite their normal mating behavior, whereas their heterozygous littermates produced pups with normal litter size (8.91 ± 0.57 pups, n = 10 breeding pairs) and intervals (1.22 ± 0.15 pups, n = 10 breeding pairs). Therefore, both *Catsper3*$^{-/-}$ and *Catsper4*$^{-/-}$ male mice are infertile.

### *Normal Spermatogenesis but Lack of Hyperactivated Sperm Motility in* Catsper3$^{-/-}$ *and* Catsper4$^{-/-}$ *Male Mice*

To define the defects underlying the infertility phenotype, we first examined the testes of *Catsper3*$^{-/-}$ and *Catsper4*$^{-/-}$ males. No differences were observed in the testis weight (Fig. 3A) and epididymal sperm counts (Fig. 3B). Consistent with similar testis weights, robust spermatogenesis was observed in both WT and *Catsper3*$^{-/-}$ or *Catsper4*$^{-/-}$ testes (Fig. 3D). We collected epididymal spermatozoa into HTF medium and examined the percentage of motile sperm every 30 min for 2 h at room temperature. Motile *Catsper3*-null sperm decreased from 55% to only 10%, and motile *Catsper4*-null sperm were reduced from 48% to 13% within 2 h, whereas WT sperm showed a slightly lowered percentage motility (67% to 51%).

In the noncapacitating HS medium (containing $Ca^{++}$ but no BSA or $HCO_3^-$), both WT and *Catsper3*-null or *Catsper4*-null spermatozoa displayed similar swimming patterns, which were characterized by symmetric tail beating in high frequency and low amplitude (termed *initial motility* herein; Fig. 4, A–C, and supplemental movies 1–3 available online at www.biolreprod.org), suggesting that maintaining initial motility does not require CATSPER3 or CATSPER4. In the capacitating HFT medium (containing $Ca^{++}$, BSA, and $HCO_3^-$), WT spermatozoa started to develop hyperactivated motility, which was characterized by asymmetrical tail beating in low frequency and high amplitude, after 30 min of incubation at room temperature, whereas *Catsper3*-null or *Catsper4*-null spermatozoa still displayed initial motility during 2 h of incubation in HTF (Fig. 4, D–F, and supplemental movies 4–6 available online at www.biolreprod.org). This result implies that functional CATSPER3 and CATSPER4 proteins are required for hyperactivated sperm motility during capacitation. Interestingly, when transferred from HTF or HS medium to a DVF solution ($Ca^{2+}$ free), WT sperm became motionless within 15 min, whereas *Catsper3*-null or *Catsper4*-null spermatozoa still displayed a weaker initial motility (Fig. 4, G–I, and supplemental movies 7–9 available online at www.biolreprod.org), suggesting that in the absence of CATSPER3 or CATSPER4 protein, sperm initial motility can be maintained when extracellular $Ca^{2+}$ is not available.

To define the swimming properties of the *Catsper3*-null and *Catsper4*-null sperm, we employed a computer-assisted sperm analysis (CASA) software and analyzed the motility parameters, including path velocity, linear velocity, track velocity, and straightness. With 30 min of incubation in HTF medium, *Catsper3*-null and *Catsper4*-null sperm showed slightly higher track velocity and path velocity than WT sperm, although the differences were not statistically significant (Fig. 3, E and F). At 2 h of incubation in HTF, however, *Catsper3*-null and *Catsper4*-null sperm displayed significantly reduced track velocity and path velocity, as well as linear velocity, whereas WT spermatozoa showed increased track velocity and path

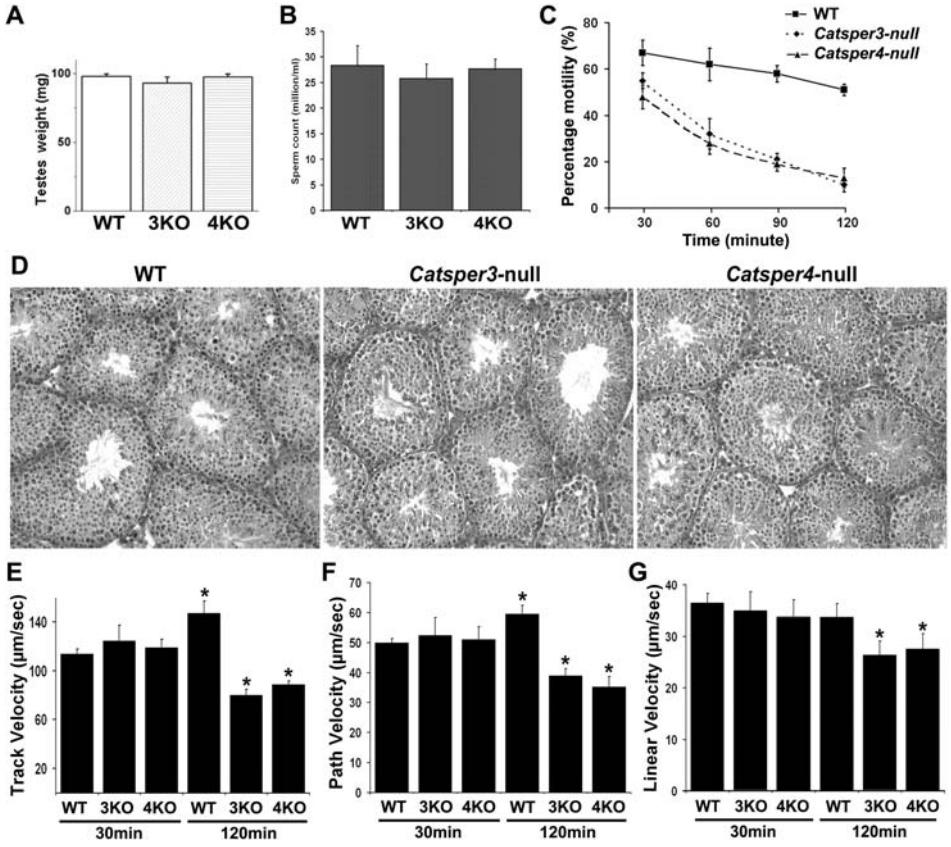


FIG. 3. *Catsper3*-null and *Catsper4*-null mice display normal spermatogenesis but defective sperm motility. **A**) Testis weights of WT, *Catsper3*-null, and *Catsper4*-null mice at ages 8–10 wk showed no significant differences (n = 8; $P > 0.05$). **B**) Sperm counts for WT, *Catsper3*-null, and *Catsper4*-null mice at ages 8–10 wk showed no significant differences (n = 8; $P > 0.05$). **C**) Percentage motility of sperm from WT, *Catsper3*-null, and *Catsper4*-null mice during a 2-h period of incubation in HTF medium at room temperature. *Catsper3*-null and *Catsper4*-null sperm displayed significantly decreased percentage motility in all time points analyzed (n = 6; $P < 0.05$). **D**) Histologies of the testes of WT, *Catsper3*-null, and *Catsper4*-null mice all show normal spermatogenesis. Original magnification ×200. **E**–**G**) CASA on track velocity (**E**), path velocity (**F**), and linear velocity (**G**) of WT, *Catsper3*-null, and *Catsper4*-null sperm in HTF medium at room temperature for 30 and 120 min. *Significant difference between the two time points analyzed for each genotype, n = 6; $P < 0.05$.

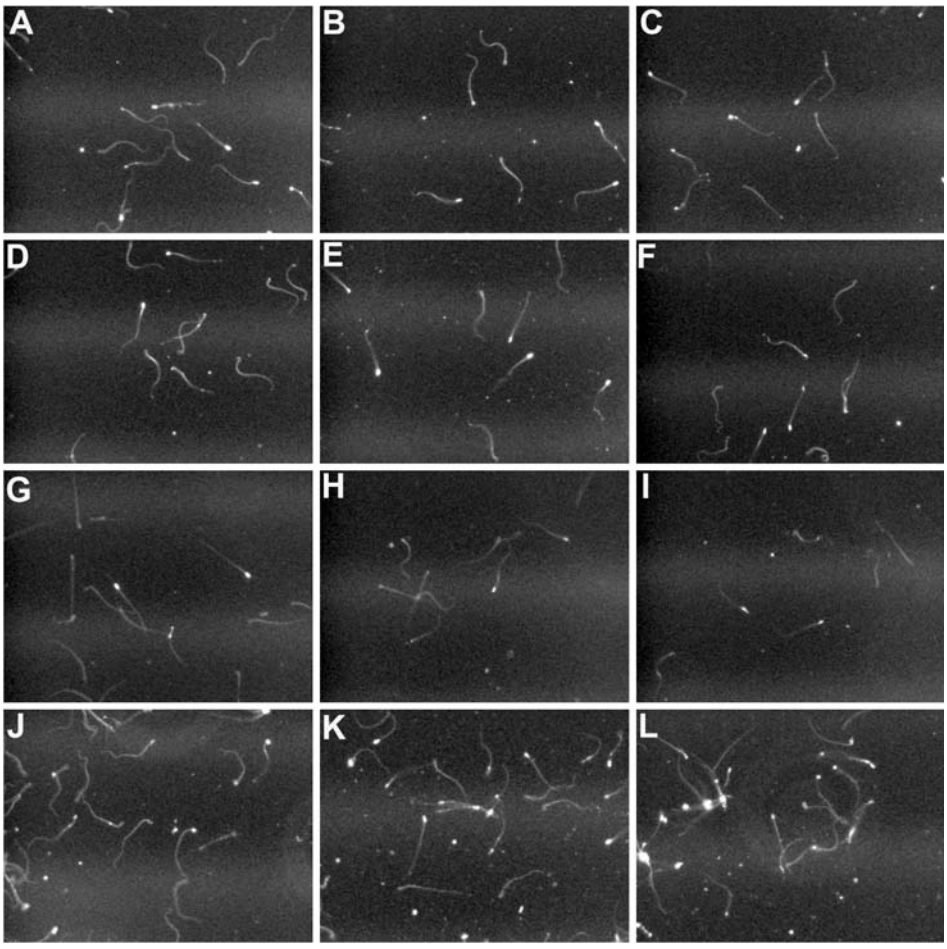


FIG. 4. Still images taken from the supplemental movies (available online at www.biolreprod.org) showing motility of epididymal sperm collected from WT (**A**, **D**, **G**, and **J**), *Catsper3*-null (**B**, **E**, **H**, and **K**), and *Catsper4*-null (**C**, **F**, **I**, and **L**) mice after incubation in the HS (**A**–**C**), HTF (**D**–**F**), DVF (**G**–**I**) or PVP (**J**–**L**) medium. Original magnification ×200.

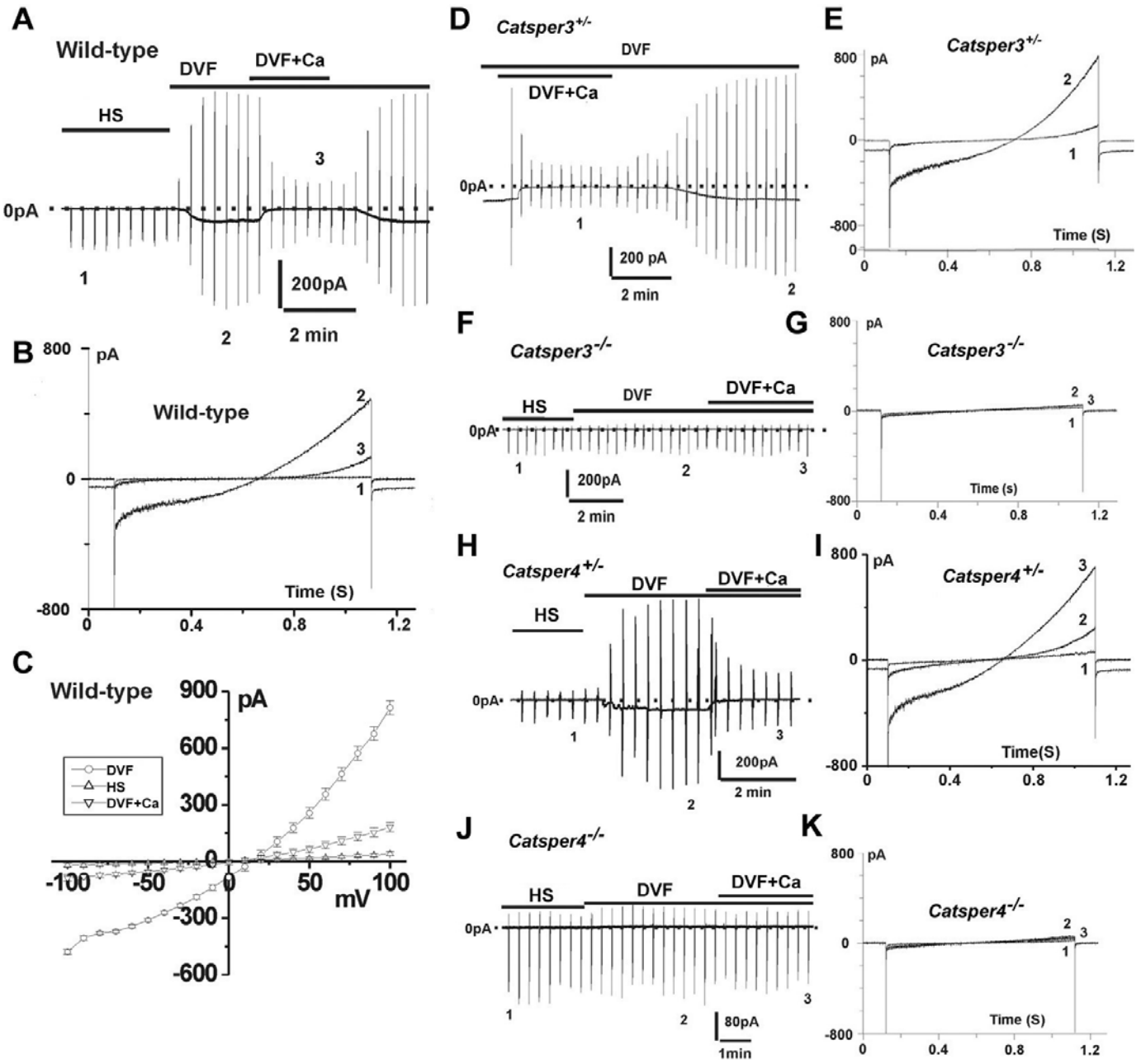


FIG. 5. Whole-cell current recordings using spermatozoa from WT, *Catsper3*$^{+/-}$, *Catsper3*$^{-/-}$, *Catsper4*$^{+/-}$, and *Catsper4*$^{-/-}$ mice. **A**) A representative whole-cell current recording using spermatozoa from WT mice in HS (1), DVF (2), or DVF + Ca (3) solution. Repetitive voltage ramp pulses (from −100 to 100 mV) were applied every 20 sec at a holding potential of 0 mV. **B**) The ramp currents under HS (1), DVF (2), or DVF + Ca (3) conditions. **C**) The current-voltage relationship under HS, DVF, and DVF + Ca conditions (n = 10; $P < 0.05$). **D**–**E**) A sustained current induced by DVF (2) was attenuated by external $Ca^{2+}$ (DVF + Ca) (1) in a spermatozoon from a *CatSper3*$^{+/-}$ mouse (**D**). The ramp currents under DVF (2) and DVF + Ca (1) conditions are shown (**E**). **F**–**G**) A representative whole-cell current under HS (1), DVF (2), and DVF + Ca (3) conditions recorded from a spermatozoon from a *CatSper3*$^{-/-}$ mouse (**F**), and the corresponding ramp currents under these three conditions (**G**). **H**–**I**) A representative whole-cell current in HS (1) or DVF (2) or DVF + Ca (3) solution recorded from a spermatozoon of a *CatSper4*$^{+/-}$ mouse (**H**), and the corresponding ramp currents (**I**). **J**–**K**) A representative whole-cell current from a sperm of a *CatSper4*$^{-/-}$ mouse under HS (1), DVF (2), or DVF + Ca (3) conditions (**J**), and corresponding ramp currents (**K**).

velocity (Fig. 3, E and F) and slightly reduced linear velocity (Fig. 3G). Manual counting revealed that ~70% WT sperm displayed hyperactivated motility, but no hyperactivated sperm were observed in *Catsper3*-null and *Catsper4*-null sperm.

To test the motility in viscous medium, we transferred sperm that had been incubated in HTF medium for 1 h to ~7% PVP in HTF. WT sperm still displayed hyperactivated motility and efficiently progressed through the viscous medium (Fig. 4J and supplemental movie 10 available online at www.biolreprod.org). In contrast, *Catsper3*-null and *Catsper4*-null sperm showing initial motility failed to progress (Fig. 4, K and L, and supplemental movies 11 and 12 available online at www.biolreprod.org).

### *Whole-Cell Current Recording Using Spermatozoa from WT,* Catsper3$^{+/-}$, Catsper3$^{-/-}$, Catsper4$^{+/-}$, *and* Catsper4$^{-/-}$ *Mice*

$Cl^-$ and $K^+$ channels have been reported to participate in the sperm acrosome reaction [28] and in sperm volume regulation

and capacitation [29–31], respectively. In the present study, effects of $Cl^-$ and $K^+$ channels were excluded by replacing $Cl^-$ with methanesulphonate and gluconate and $K^+$ with $Cs^+$. In HS solutions, whole-cell currents of WT spermatozoa showed a very low conductance (Fig. 5A). After exposure of sperm cells to DVF solutions for 2.8 ± 0.3 min (n = 10), a sustained inward current at a holding potential of 0 mV was developed, during which repetitive voltage ramp pulses (from 100 mV to −100 mV, 0.2 V/sec) were applied every 20 sec (Fig. 5A). While the holding currents were increased from −0.5 ± 0.4 pA to −74.1 ± 4.1 pA, the inward current at the membrane potential of −100 mV was potentiated from −20.6 ± 9.2 pA to −479.5 ± 13.0 pA, and the outward current at the membrane potential of 100 mV was elevated from 40.4 ± 10.3 pA to 813.7 ± 36.8 pA (n = 10; P < 0.05; Fig. 5, B and C). However, upon an addition of 33 μM $Ca^{2+}$ to DVF (DVF + Ca), the current induced by DVF was significantly attenuated (Fig. 5, B and C). These findings are consistent with a previous study [32], suggesting that free extracellular $Ca^{2+}$ has an inhibitory effect on the DVF-activated current.

Similarly to WT sperm, the DVF-induced currents could be readily detected in spermatozoa of the *Catsper3*$^{+/-}$ mice, and an application of the DVF + Ca solution inhibited this current, which could be recovered after returning to the DVF condition (Fig. 5, D and E). The amplitude of the currents under HS, DVF, and DVF + Ca conditions were −22.9 ± 9.8 pA, −449.8 ± 10.9 pA, and −83.7 ± 16.5 pA at the membrane potential of −100 mV, and 37.4 ± 10.9 pA, 842.6 ± 39.0 pA, and 181.0 ± 26.5 pA at the membrane potential of 100 mV, respectively (n = 9; $P < 0.05$). However, in *Catsper3*-null sperm, the DVF-activated whole currents were undetectable. The holding current and current amplitude under HS, DVF, and DVF + Ca conditions remained unchanged (Fig. 5F). The ramp currents under these conditions showed no differences (n = 9; $P > 0.05$; Fig. 5G). This finding suggests that this DVF-induced cationic current may pass through the CATSPER3 channel.

Similarly to spermatozoa of WT and *Catsper3*$^{+/-}$ mice, exposure of spermatozoa collected from *Catsper4*$^{+/-}$ mice to the DVF solution evoked a sustained inward current at the holding potential of 0 mV (Fig. 5, H and I). This current was significantly inhibited in the DVF + Ca solution (Fig. 5, H and I). The amplitudes of the currents under HS, DVF, and DVF + Ca conditions were −25.8 ± 10.6 pA, −453.8 ± 12.4 pA, and −86.7 ± 18.9 pA (at −100 mV), and 39.3 ± 11.4 pA, 852.5 ± 43.9 pA, and 194.6 ± 29.5 pA (at 100 mV), respectively (n = 9; $P < 0.05$). However, the whole-cell current in *Catsper4*-null spermatozoa was undetectable under either DVF or DVF + Ca conditions (Fig. 5, J and K). The ramp currents in each condition showed no significant differences between *Catsper4*-null and *Catsper3*-null spermatozoa (n = 9; $P > 0.05$). The monovalent currents carried by $Na^+$ in the inward direction and by $Cs^+$ in the outward direction can only be detected when both CATSPER 3 and 4 channel(s) are present in spermatozoa (WT, *Catsper3*$^{+/-}$, and *Catsper4*$^{+/-}$; Fig. 5, B, C, E, and I), suggesting that these DVF-induced cationic currents may pass through the CATSPER 3 or 4 channel(s). Therefore, this channel has been termed $I_{CatSper}$ [32].

## DISCUSSION

Both *Catsper3*$^{-/-}$ and *Catsper4*$^{-/-}$ mice are completely infertile, despite their normal sperm counts and initial motility. The infertility can be ascribed to three major defects. First, a quick loss of motility (to ~10% within 2 h in capacitating medium HTF) would significantly reduce the number of *Catsper3-null* and *Catsper4-null* spermatozoa that can reach the eggs in the ampulla region of the oviduct. This finding suggests that functional CATSPER3 and CATSPER4 proteins are required for maintaining the motility and probably also for the survival of spermatozoa. Second, the inability of *Catsper3-null* and *Catsper4-null* spermatozoa to penetrate the viscous medium suggests that these mutant sperm cannot progress efficiently in the mucosa of the female reproductive tract, and thus the number of sperm reaching the eggs would be reduced. Third, a lack of hyperactivated motility compromises the ability of the mutant sperm to generate sufficient forces to penetrate cumulus cells and zona pellucida during fertilization.

The sperm motility defects in *Catsper3*$^{-/-}$ and *Catsper4*$^{-/-}$ mice are very similar to those seen in *Catsper1*$^{-/-}$ and *Catsper2*$^{-/-}$ mice. Lack of any of the four CATSPER proteins appears to result in deficiency in hyperactivated motility and quick loss of initial motility in the capacitating conditions. An almost identical phenotype in mice lacking either of the four CATSPER channel proteins, their highly homologous domain structure, and their similar localization to the sperm flagellum all suggest that CATSPERs 1–4 form a tetramer cation channel, which is required for the development of hyperactivated motility during sperm capacitation in the female reproductive tract. Interestingly, Qi et al. reported recently that the four CATSPER proteins are indeed associated with each other and form a tetramer [33]. In addition, similar sperm motility defects in the *Catsper3* and *Catsper4* knockout mice were also reported [33, 34].

Approximately 40%–80% of male infertility patients are idiopathic [35]. A significant portion of idiopathic infertile men display normal sperm counts and normal initial motility [35]. Since the majority of the andrology laboratories rely on CASA, which may not be programmed properly to detect sperm hyperactivated motility, this type of subtle defect may be missed. Diagnosis of defects in sperm hyperactivated motility can be achieved easily if microscopic observation under higher magnification (20× or 40×) can be performed on sperm incubated in the capacitating medium. The four CATSPER proteins display high degrees of sequence conservation between mice and humans [23, 36], suggesting that the human CATSPER proteins may have a similar function in the regulation of hyperactivated sperm motility. Supporting this notion, Nikpoor et al. have shown that *CATSPER1* gene expression is reduced in infertile patients with defective sperm motility [37]. Further genetic screening for patients with defects in hyperactivated sperm motility will significantly increase the chance of finding mutations in any of the four *CATSPER* genes. For patients with null mutation in any of the four *CATSPER* genes, conventional IVF procedure should not be recommended, and intracytoplasmic sperm injection may be the only choice for fertilization.



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